Rajender Singh *Editor*

Genetic Testing in Reproductive Medicine

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Preface

In the post-human genome sequencing era, the availability of human genome sequence has made genetic testing feasible. This book illustrates the importance and significance of molecular genetic testing in reproductive disorders. After the sequencing of the human genome was completed, genetic association studies identified a number of plausible and definitive genetic causes of various human disorders. Mass genotyping data or targeted sequencing has identified potentially pathogenic mutations for a number of human diseases. While there is evidence of genetic elements in almost all reproductive disorders, genetic testing in clinical settings is in practice for only a few disorders. The present book covers the genetic etiology of various reproductive system anomalies, including disorders of sexual development, male infertility, female infertility (PCOS and POF), recurrent pregnancy loss, pre-term birth, endometrial cancer, cervical cancer, and other related disorders. In addition, it covers reproductive career screening, preimplantation genetic testing, prenatal genetic testing, and endometrial receptivity for better care of reproductive health issues. Microbiome testing and its contribution to genetic testing in reproductive medicine, which is in infantile stage, has also been discussed. Genetic testing for some of these issues has already come into practice, genetic etiology for others is established but not clinically practiced, and genetics of yet others is in infancy due to limited data availability. For the disorders where sufficient genetic data to undertake genetic testing is not available, we have tried to put together the evidence for proposing such a test in the near future. Apart from this, the book also covers ethical, moral, and technical issues in genetic testing in reproductive medicine. The book aims to set a standard with regard to genetic testing in the reproductive medicine field and encourage further data generation in the areas of lacunae. The book aims to serve students, researchers, clinicians, and professors in the field of reproductive biology.

Acknowledgments

The book was conceived while discussing the future of genetic testing with some scientist friends. The same evening, I checked the status of genetic testing in reproductive medicine. Looking at immense possibilities in the field and very limited clinical usage, I proposed this book on *Genetic Testing in Reproductive Medicine*. I am grateful to all contributors for their acceptance of submitting book chapters and their timely submission for publication. This is my third book, and the excitement to write it was driven by the success of the first two books in this field. I am grateful to the Council of Scientific and Industrial Research (CSIR) for providing adequate research facilities. I also acknowledge the indirect contributions of other funding agencies, such as the Indian Council of Medical Research (ICMR), the Department of Biotechnology (D.Bt.), and the Department of Science and Technology (DST), for funding our research in reproductive biology. I am grateful to the numerous patients and donors who donated samples for our research. Some of the findings cited in this book are from our studies conducted over the last two decades.

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Rajender Singh is a Senior Principal Scientist and Professor at the Central Drug Research Institute (CDRI), Lucknow, India. He has been working as a scientist at this institute for the last 16 years. He has 20 years of experience in reproductive genetics and genomics. His research interests lie in the genetics and genomics of male reproduction and infertility, with specific emphasis on sperm RNAs, genetics, and epigenetics. Dr. Singh has published more than 164 research articles and 36 book chapters. Dr. Singh is serving as an associate editor for Reproductive Biology and Endocrinology, Reproductive Health, and Andrologia, and as the speciality chief for the Andrology section of Frontiers in Reproductive Health journals. His work has been recognized by a number of prestigious scientific bodies in the form of the Haryana Yuva Vigyan Ratna Award (2018), the INDO US Post-doctoral Research Fellowship at the University of Alabama at Birmingham (2017), the Innovative Young Biotechnologist Award from D.Bt (2015), the Young Scientist Award from the Council of Scientific and Industrial Research (2014), the Young Scientist Award from the Indian Science Congress Association (2012), the Young Scientist Award from the Indian National Scientific Academy (2011), and the Young Scientist Award from the Indian Society of Human Genetics (2006). He is a member of the Indian Society for the Study of Reproduction and Fertility (ISSRF), the Society for Reproductive Biology and Comparative Endocrinology (SRBCE), the Indian Fertility Society (IFS), and the Indian Society of Human Genetics (ISHG).

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Genetic Testing in Disorders of Sexual Development

1

Vertika Singh, Ankur Saharan, David Handelsman, and Rajender Singh

Abstract

Disorders of sex development (DSD) present in a variety of forms, ranging from normal-looking male or female external genitalia and phenotypes to completely reversed genitalia and phenotypes, which contrast with the chromosome complement of the individual. The underlying reasons could be chromosomal disorders, gene dosage, gene mutations, or exposures to high doses of the cross-sex hormones. Chromosomal DSDs due to aneuploidies include Turner syndrome (45, X) and Klinefelter's syndrome (47, XXY), whose presentation may also be mosaic. 46, XY DSD may occur due to *SRY* mutations (Swyer syndrome) and *SOX9* mutations (campomelic dysplasia). Other genes causing 46, XY DSD are *GATA4*, *SOX8*, *DMRT1*, *DMRT2*, and *DHH* as well as translocation of the *SRY* gene to an X chromosome. Modified testosterone synthesis or action may occur due to mutations in the *AR*, *NR5A1*, *CYP17A1*, *POR*, and *SRD5A2* genes. Mutations in the androgen receptor gene may result in a spectrum of androgen insensitivity phenotypes. The occurrence of 46,XX DSD can be attributed to mutations in the *CYP21A2*, *CYP11B1*, *CYP19A1*, *HSD3B2*, *WNT4*, and *FOXL2*

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genes. Ovotesticular dysgenesis may arise due to mutations in the *RSPO1*, *WNT4*, *SOX3*, *NR5A1*, *NR0B1*, *AMH*, and *AMHR2* genes. When considering a genetic diagnosis, chromosomal analysis serves as the initial step in analyzing DSD genetics, as the identification of aneuploidies often eliminates the need for additional analysis in many cases. Once aneuploidy is ruled out, additional analysis becomes necessary to identify gene mutations and/or copy number variations, which varies depending on the specific type of disorder.

Keywords

Disorders of sexual differentiation · Gonadal trans-differentiation · Turner syndrome · Klinefelter's syndrome · Gonadal dysgenesis · Genetic testing · Reproductive medicine

1.1 Introduction

With the arrival of a new baby, the first question usually asked is, "Is it a boy or a girl?" However, it is unexpected for the parents as well as the clinicians to find a newborn baby with ambiguous external genitalia. Ambiguous genitalia, apart from raising concerns regarding the sexual rearing of the newborn, can rarely (1 in 15,000 newborns) be the indication of a life-threatening condition, such as congenital adrenal hyperplasia (REF). Therefore, children born with ambiguous genitalia should be evaluated by a multidisciplinary team of experts to treat the condition and gender assignment. Certain individuals may present with typical male or female genitalia at birth but encounter challenges in the development of secondary sexual characteristics. On the other hand, some individuals may exhibit normal external genital anatomy and seemingly normal progression of secondary sexual development, only to later discover issues related to infertility, leading to broader concerns about their overall development. Thus, disorders of sexual development (DSD) present an array of phenotypes, from apparently normal-looking external genitals to completely sex-reversed external genitalia and phenotypic development. Older, now outdated terminologies with pejorative tone for these disorders such as hermaphrodite and pseudohermaphrodite are no longer used. However, as these disorders are complex in their nature and these terminologies were confusing for urologists (Kim and Kim 2012), in 2005, multidisciplinary meetings of clinicians and researchers were recommended with a preferred nomenclature and replaced the terms such as hermaphroditism and pseudohermaphroditism with the disorders of sexual development (DSDs) (the Chicago Consensus) (Houk et al. 2006; Lee et al. 2006; Kim and Kim 2012).

Sexual development is a complex, multistep process that involves an intricate interplay of genetic and molecular cues that direct a bipotential gonad to develop into the binary form of either testes or ovaries. This process is called sex determination. Sex determination is followed by the differentiation of internal ducts and external genitalia, which is termed sex differentiation (Rey et al. 2016). The process of sex

determination and differentiation is under strict regulation by various genetic and nongenetic factors, alterations in which may result in disorders of sexual development (DSD) (Kim and Kim 2012; Witchel 2018). DSD comprises an array of abnormalities that vary in frequency and severity. Some of these abnormalities, when presented in their mildest form, such as the mild androgen insensitivity syndrome, may not affect gonadal or sexual functionality.

A lot of progress has been made with regard to the genetics of DSDs comprising genetic, epigenetic, and hormonal factors during prenatal and postnatal development; however, the factors responsible for all DSDs are far from understood. Genetic testing in DSDs constitutes an integral and important component of clinical workup, as the genetic makeup of the individual is critical to deciding whether the individual should be raised as a male or a female and, in a minority of cases, to managing what may become a medical emergency. In this chapter, we will discuss the genetic basis of the disorders of sexual development and the current scenario of the genetic diagnosis of these disorders.

1.2 Genetics of Sexual Differentiation and Development

The development of sexual characteristics is intricately regulated by a multitude of activating and inhibiting factors, which precisely control the spatial and temporal expression of genes involved in sexual development. The complicated and multilayered regulation is resilient to minor alterations due to the biological redundancy implied to ensure the critical process of species propagation. The initial differentiation of a bipotential gonad involves a complex interplay of various genes, some of which include the sex-determining region of the Y-chromosome (*SRY*), androgen receptor (*AR*), empty spiracles homeobox 2 (*EMX2*), LIM homeobox factor 9 (*LHX9*), Wilms' tumor 1 (*WT1*), chromobox homolog 2 (*CBX2*), steroidogenic factor 1 (*NR5A1*), sine oculis-related homeobox 1/4 (*SIX 1/4*), and GATA binding protein 4 (*GATA4*) (Tanaka and Nishinakamura 2014) (Fig. 1.1). The differentiation of a bipotential gonad/ridge into male or female gonads involves complex regulation, ending in the activation of one of the binary pathways. This implies that testicular development suppresses the ovarian pathway and vice versa. The development of the ovaries is considered the "default pathway" of differentiation, and the presence of the SRY gene is believed to redirect this default pathway toward male development (Tanaka and Nishinakamura 2014).

Around 4–6 weeks of gestation, the urogenital ridge arises as an outgrowth of the coelomic epithelium. Subsequently, the urogenital ridge gives rise to the kidneys, adrenal cortex, gonads, and reproductive tract. Activation of the SRY gene triggers the differentiation of a bipotential gonad into a testis. It induces and acts upon its receptor SOX9, which in turn ramifies the testis differentiation (Tanaka and Nishinakamura 2014; Witchel 2018). Upon the differentiation of Sertoli cells, the developing testis gets organized into two compartments: one compartment consisting of germ cell proliferation to ultimately form spermatozoa surrounded by the Sertoli cells and enclosed by the peritubular myoid cells, forming testicular

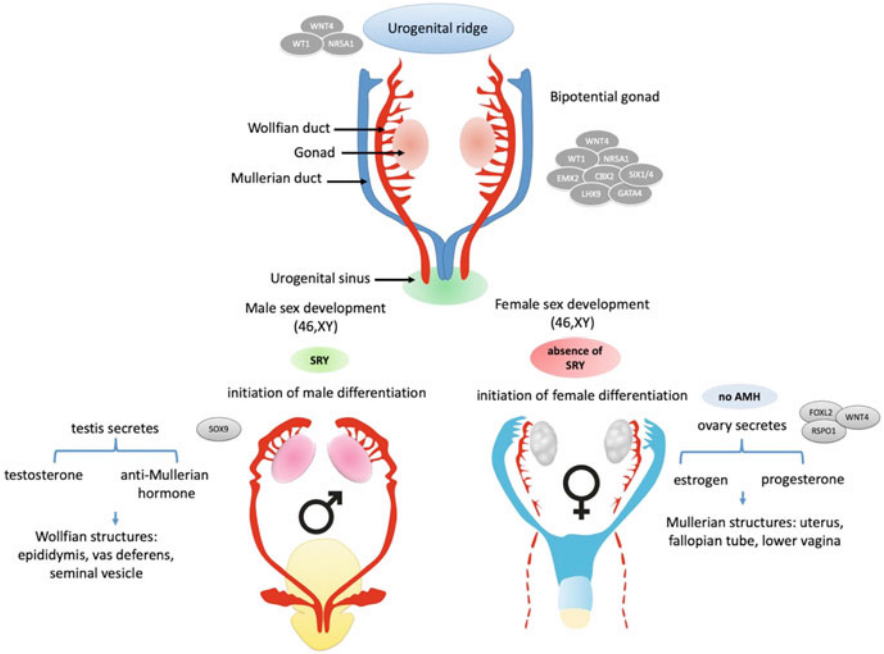


Fig. 1.1 Diagrammatic representation of the major pathways of male and female sexual differentiation

cords, and the other compartment consisting of the testis interstitium, which contains Leydig cells and testis vasculature (Witchel 2018).

Gonadal development begins with the development of both the Wolffian and Müllerian ducts. Testosterone secreted by the fetal Leydig cells stabilizes the Wolffian ducts, directing the development of the epididymis, vas deferens, ejaculatory duct, and seminal vesicle. Testosterone regulates and promotes the testicular descent into the scrotum, which is completed around 32 weeks of gestation. Insulin-like factor 3 (INSL3) is another hormone secreted by the testis that promotes the descent of the testis into the scrotum, while anti-Müllerian hormone secreted by the Sertoli cells induces the regression of the Müllerian ducts (Witchel 2018).

In the absence of SRY, ovarian differentiation unfolds by default. The differentiation of the ovary is initiated and regulated by Forkhead transcription factor 2 (FOXL2), R-Spondin 1 (RSPO1), wingless-type MMTV integration site family member 4 (WNT4), and the beta catenin pathway (Biason-Lauber 2012) (Fig. 1.1). In the absence of androgen action from testosterone and dihydrotestosterone (DHT), female external genital structures of the clitoris, vagina, and labia develop.

Interestingly, the differentiation of a germ cell into spermatogonia or oogonia does not depend on the XX or XY karyotype. Rather, germ cell differentiation is induced by neighboring somatic cells under the influence of high levels of retinoic acid (RA), a meiosis-inducing factor. For murine female germ cells at 12.5 dpc, RA

induces Stra8 (stimulated by retinoic acid 8) expression, which initiates meiosis in female germ cells. However, for the developing testis, CYP26B1, a RA metabolizing enzyme, prevents meiosis in male PGCs, which resumes after birth when a subset of spermatogonia are exposed to RA (Busada et al. 2014). In this process, testosterone acts on the mature Sertoli cells via androgen receptor, which facilitates the completion of meiosis and further development to complete the process of spermiogenesis (Smith and Walker 2014) and spermiation (Holdcraft and Braun 2004). AR in Sertoli cells expresses in a stage-specific manner to regulate spermatogenesis (Holdcraft and Braun 2004).

1.3 Disorders of Sexual Development

Sex determination and differentiation occur at three levels: chromosomal, gonadal, and secondary sexual differentiation. Disruptions at any of these stages can lead to a variety of disorders known as disorders of sexual development (DSDs), which vary depending on the specific molecular aberration and the stage of development. DSD is characterized by a mismatch between an individual's chromosome complement or genetic material and the appearance of their genitals. The estimated frequency of DSD is approximately 1 in 5500 births. DSD encompasses a broad spectrum of phenotypes and can be classified into categories based on the sex chromosome complement, including aneuploidies, 46, XX DSD, 46, XY DSD, and rare mosaic combinations.

1.3.1 Sex Chromosome DSD

Sex chromosome aneuploidies, such as Turner's syndrome (45, X) or Klinefelter's syndrome (47, XXY), both sometimes mosaic, constitute sex chromosome DSDs. The prevalence of Turner syndrome is about 1 in 2500 live-born females (Nielsen and Wohlert 1991). Turner syndrome girls typically present with a short, webbed neck, low-set ears, a broad chest with widely spaced nipples, a low hairline at the neck, puffy hands and feet, and soft nails that turn upward, often accompanied by slow statural growth. A standard 30-cell karyotype analysis is required for the diagnosis of Tuner syndrome; however, for the diagnosis of mosaic, fluorescence in situ hybridization (FISH) is performed (Witchel 2018). A mosaic Turner syndrome female including some Y chromosome material may also display some features of virilization.

Klinefelter's syndrome is characterized by chromosomal complement 47, XXY with a prevalence of around 1 in 500 males (Witchel 2018). These males display characteristics such as eunuchoidal tall stature (due to delayed estrogen-mediated epiphyseal fusion), small testicles and penis, delayed puberty, poor musculature, hypogonadism, gynecomastia, and infertility (azoospermia).

1.3.2 46,XY DSD

46, XY DSD is characterized by impaired androgenization due to defects in testis differentiation, androgen synthesis, or androgen action. This category includes patients presenting with abnormal testicular differentiation and abnormal secondary sexual development. The genetic etiology of 46,XY DSD includes loss-of-function mutations in the *SRY* or *SOX9* gene, leading to gonadal dysgenesis. Mutations in the SRY gene lead to Swyer syndrome, which is characterized by 46,XY complete gonadal dysgenesis. Children with Swyer syndrome do not present with short stature or Turner's characteristics but show typical female secondary sexual characteristics and some internal female reproductive structures, such as a small uterus. However, they fail to menstruate, and a large number of them are identified by clinical investigations due to failure of menarche.

SRY, in conjunction with NR5A1 (SF1), activates the expression of the downstream signalling molecule SOX9, which is responsible for driving testis differentiation (Sekido and Lovell-Badge 2008). Mutations in the *SOX9* gene lead to campomelic dysplasia, an autosomal form of dwarfism commonly associated with 46,XY DSD in affected individuals. The phenotype observed in these individuals results from the failure of testicular development and a lack of androgen action. The extent of phenotypic variation depends on the type and location of the mutation within the *SOX9* gene, which determines the residual activity of the gene. Campomelic dysplasia, a frequently fatal syndrome, can be diagnosed through ultrasound examination based on the shortened length of long bones. Mutations in the *GATA4* gene, encoding a transcription factor essential for gonadal development and anti-Müllerian hormone (AMH) synthesis or action, also contribute to a variant form of the 46,XY DSD phenotype.

Other genes associated with XY gonadal dysgenesis include *SOX8* (SRY-box transcription factor 8), DHH (desert hedgehog signaling molecule), *DMRT1* (doublesex and mab-3-related transcription factor 1), and *DMRT2* (doublesex and mab-3-related transcription factor 2) (Yatsenko and Witchel 2017; Mehta et al. 2021). Additional gene mutations that impact testosterone synthesis include *NR5A1* (nuclear receptor subfamily 5 group A member 1), *CYP17A1* (cytochrome P450 family 17 subfamily A member 1), *POR* (cytochrome P450 oxidoreductase), and impaired conversion of testosterone to the more potent androgen DHT by SRD5A2 (steroid 5 alpha-reductase 2).

Mutations in the HSD17B3 gene may lead to a deficiency in the enzyme 17β-HSD3, affecting the conversion of androstenedione to testosterone and causing decreased testosterone levels and reduced virilization of males (Donadille et al. 2018). 5α-reductase, encoded by the SRD5A2 gene, catalyzes the conversion of testosterone to dihydrotestosterone (DHT). Mutations in this gene can lead to impaired enzyme function, resulting in decreased levels of DHT and subsequent disruptions in sexual development in genetically male individuals. *SRD5A2* mutations result in a variety of clinical manifestations in affected individuals, such as ambiguous genitalia, underdeveloped or feminized external genitalia, and incomplete masculinization. In certain cases, individuals with SRD5A2 mutations may be

raised as females due to the appearance of their external genitalia. The phenotype can vary based on the specific mutation, its location, and other genetic and environmental factors.

Several studies have investigated the association between *SRD5A2* mutations and DSD. For example, Wilson et al. (1993) identified mutations in the *SRD5A2* gene in individuals with 5-alpha-reductase deficiency, a form of DSD characterized by ambiguous genitalia and incomplete virilization in genetically male individuals. Similarly, Imperato-McGinley et al. (1992) conducted studies on a population with a high prevalence of *SRD5A2* mutations, known as the "Guevedoce" population, and provided insights into the effects of these mutations on sexual development. Additionally, a recent study by Bose et al. (2022) reported several cases of SRD5A2 deficiency in individuals with 46 XY DSD, highlighting the diverse phenotypic presentations observed in affected individuals. The study described the challenges in diagnosis, emphasizing the importance of a comprehensive evaluation involving clinical assessment, hormone analysis, genetic testing, and imaging studies.

If testosterone production is normal, some testosterone (T) gets converted into dihydrotestosterone (DHT), and both T and DHT act via androgen receptor to facilitate sexual differentiation. The T-receptor complex plays a pivotal role in various biological processes, including the differentiation of Wolffian ducts during the embryonic stage, the regulation of luteinizing hormone secretion by the hypothalamus-pituitary-gonadal axis, and spermatogenesis. The DHT-receptor complex plays a crucial role in facilitating the development of urogenital sinus derivatives to form the external genitalia and prostate gland during the embryonic stage, with further maturation occurring during puberty. Mutations in the androgen receptor (AR) gene lead to a spectrum of androgen insensitivity syndrome (AIS), resulting in a range of phenotypes. These AR mutations cause varying degrees of loss of androgen function, leading to altered male phenotypes, ranging from nearly normal male phenotype to typical female phenotype. In the latter case, individuals exhibit normal female external genitalia, the absence of ovaries, and either a vestigial or absent uterus (Rajender et al. 2007; Gottlieb and Trifiro 2017). Complete androgen insensitivity syndrome (CAIS) individuals present with a typical female phenotype, including breast development, but experience infertility, unable to conceive or bear children (Singh et al. 2006; Rajender et al. 2009). Partial androgen insensitivity syndrome (PAIS) individuals may have ambiguous external genitalia (Singh and Ilyayeva 2023), while those with mild androgen insensitivity syndrome (MAIS) may exhibit under-masculinization or solely male infertility within an otherwise normal male phenotype (Thangaraj and Rajender 2009).

1.3.3 46,XX DSD

46,XX DSD refers to conditions where females have ovarian maldevelopment or adrenal hyperandrogenism. Congenital adrenal hyperplasia (CAH) is the most common condition associated with 46, XX DSD (Yau et al. 2019). CAH is a disorder of steroidogenesis that occurs by far most commonly due to the complete or severe loss

of the 21-hydroxylase enzyme (Yau et al. 2019) due to mutations in the *CYP21A2* (cytochrome P450 family 21 subfamily A member 2) or less commonly *CYP11B1* (cytochrome P450 family 11 subfamily B member 1) genes. In the most frequent form, an affected girl usually presents with virilization of the external genitalia (>90%) (Witchel 2018), but other rarer variants may develop hypertension (11-hydroxylase). Affected boys with untreated 21-hydroxylase deficiency often develop premature virilization causing short stature due to the premature epiphyseal closure. Sometimes, the virilization is so severe that the affected genetically female individuals with severe 21-hydroxylase deficiency are exposed to excess androgens and may present with virilized external genitalia (White and Speiser 2000). Mutations in certain genes, such as *CYP19A1* and 17 *HSD3B2*, are also linked to disorders of sexual development (DSD). *CYP19A1* mutations can cause aromatase deficiency, resulting in reduced estrogen levels and an excess of androgens. This imbalance disrupts normal sexual development.

Loss-of-function mutations in the genes encoding ovarian factors may result in ovarian dysgenesis or premature ovarian failure. There is only one report of a *WNT4* mutation in a patient with affected gonadal differentiation (ovotestis), renal dysgenesis, and adrenal and pulmonary deformities, which the authors named "SERKAL syndrome." The individuals in this report presented ovotesticular gonadal differentiation with varying level of external genitalia virilization (Mandel et al. 2008). Nevertheless, the absence of further reports on *WNT4* mutations is likely attributable to their embryonic lethal nature. In a similar vein, the controlled expression of FOXL2 is vital for maintaining ovarian function. Loss of FOXL2 expression in mice results in premature ovarian insufficiency (POF) and defects in follicular development (Uda et al. 2004), while in goats, it has been associated with the "trans-differentiation" of the gonad from ovary to testis (Boulanger et al. 2014). Rare mutations, unlikely to be significant contributors to POF, have been reported in humans (Harris et al. 2002; Chatterjee et al. 2007); nevertheless, there have been no reported cases of 46,XX impaired gonadal differentiation associated with *FOXL2* mutation in humans. While Alfred Jost's theory initially proposed a passive development of ovaries, subsequent studies have revealed the involvement of active mechanisms in ovarian differentiation. However, mutations in other genes involved in ovarian differentiation, such as *RSPO1*, *CTNNB1*, and *FST*, have not been reported in human cases of 46,XX DSD.

1.3.4 Other Forms of DSDs

Besides these, patients with ovotesticular disorders are characterized by the presence of both seminiferous tubules and ovarian follicles in the same individual. That rare class of DSD is presented by 46,XX males (but usually *SRY* positive due to translocation of the *SRY* gene) with an incidence of 1:20,000 newborn males. Clinical presentation in these individuals includes some degree of virilization according to the degree of *SRY* gene expression causing a variable degree of testicular differentiation, leading to virilization of the external genitalia manifest as

either ambiguous or a range of typical but often subnormal male genitalia in conjunction with the absence of Mullerian ducts or other syndromic features (Anık et al. 2013). About 15% of these individuals are identified at birth because of ambiguous genitalia, and the remaining 85% are identified at puberty because of gynecomastia and azoospermia, but they often have pubic hair, normal penile size but small testis (Anık et al. 2013).

Male individuals with a chromosomal composition of 46,XX can be categorized into two groups based on the presence or absence of the SRY gene: SRY-positive (SRY + ve) and SRY-negative (SRY-ve) cases. The cases with the SRY gene (~90%) can be explained relatively easily by translocation of the SRY gene to another chromosome, while the etiology of those without it (~10%) remains unknown (Anık et al. 2013). The latter are likely to arise by mechanisms such as SOX9 receptor duplication or other similar post-receptor mechanisms (Vetro et al. 2015; Shankara Narayana et al. 2017).

Gene duplication of SOX9 or its regulatory elements may be associated with 46, XY DSD or 46,XX ovotesticular DSD (Grinspon and Rey 2016; Shankara Narayana et al. 2017). Some other genes associated with ovotesticular DSD in mice include *RSPO1*, *WNT4*, *SOX3*, and *NR5A1* (Swartz et al. 2017). Furthermore, gene duplication at the Xp21.2 locus harboring the *NR0B1* (nuclear receptor subfamily 0 group B member 1) gene has been associated with 46,XY gonadal dysgenesis (Bardoni et al. 1994); however, its mutations or deletions cause X-linked adrenal hypoplasia congenita, which is presented in 46,XY boys early in childhood as adrenal insufficiency and hypogonadotropic hypogonadism (Suntharalingham et al. 2015).

1.4 Genetic Testing in DSDs

Genetic testing in DSDs has made much progress over recent decades, providing a reasonable understanding of the genetic etiology of many of these disorders. It plays a crucial role in determining the genotype, explaining the phenotype and providing tailored treatment options and counseling to affected patients and their families. When a patient presents with a suspected DSD, an initial genetic together with thorough endocrine and imaging workup is necessary to rule out life-threatening adrenal disorders, such as CAH (Arboleda et al. 2013). Some of the genetic tools utilized for the diagnosis of DSDs include karyotyping, chromosomal microarrays, genome-wide association studies (GWAS), and next-generation sequencing.

1.4.1 Cytogenetic Analysis

Cytogenetic analysis plays a crucial role in the diagnosis and classification of disorders of sexual development (DSDs). Karyotyping, as recommended by Lawson Wilkins, is the primary test used to classify DSDs. Conventional karyotyping methods, such as G-banding, are employed to identify abnormalities in the chromosomal complement. To rule out mosaicism, at least 30 cells are typically analyzed

(Swansbury 2003). Fluorescence in situ hybridization (FISH) is another method used to detect chromosomal mosaicism using specific probes for X and Y centromeres. SRY-specific FISH probes can confirm the presence of the SRY gene and detect Yp rearrangements. FISH is also useful in identifying unknown marker chromosomes and Y chromosome material, aiding in assessing recurrence risk. However, FISH has limitations, such as its resolution being restricted to chromosomal rearrangements over 10 MB in size. Despite these limitations, FISH remains the most commonly used method for karyotyping and Y chromosome detection in DSD cases. In the future, greater application of more powerful and sensitive genomics through next-generation sequencing of either exomes or whole genome is likely to supplant these tests.

1.4.2 SNP Arrays, Comparative Genomic Hybridization, and Next-Generation Sequencing

As the technology used in FISH is technically cumbersome with low resolution, a wider array of different technologies aiming to detect smaller variations is investigated. SNP arrays and the comparative genomic hybridization technique offer the detection of submicroscopic gene variations. The use of customized CGH, on the other hand, allows the detection of multiple genes simultaneously, reducing the burden of individual gene testing and accelerating the diagnostic process and timely management of the case. One of the caveats of CGH is that it may fail to detect balanced chromosomal translocations (Witchel 2018). The array CGH approach has been widely employed by researchers to detect rearrangements in gonadal genes, including NR0B1/DAX1, DMRT1, SOX9, and GATA4 (Ledig et al. 2010). In addition to array CGH, multiple ligation-dependent probe amplification (MLPA) is another technique utilized to simultaneously investigate multiple genomic loci, typically around 40 loci, at once (Stuppia et al. 2012). MLPA is a cost-effective PCR-based technique, particularly useful for screening common copy number variations (CNVs) associated with disorders of sexual development (DSDs). It serves as a valuable tool for validating results obtained from array CGH. MLPA enables clinicians and researchers to easily identify novel genes and genetic loci associated with DSD, offering greater insight into the underlying genetic mechanisms involved.

The use of next-generation sequencing (NGS) methods offers a hypothesis-free approach for the identification of novel genes/variations associated with DSDs. Recently, a group of researchers utilized conventional cytogenetics, FISH, and MLPA to identify the genetic causes of DSDs in a cohort of 43 Colombian patients (García-Acero et al. 2020). MLPA targeted the analysis of the *SF1*, *DAX1*, *SOX9*, *SRY*, and *WNT4* genes. The analysis reported that 40% of patients had gonadal development disorders, 21% of patients showed 46,XY testicular DSD, and 7% of patients showed 46,XX ovarian DSD. The remaining 33% cases could not be classified into a specific class of gonadal development and were classified as

"others." Thus, genetic analysis offers a broad but incomplete spectrum approach for the etiological diagnosis of DSDs (García-Acero et al. 2020).

NGS methods provide a tantalizing possibility of their utilization in the identification of genetic mutations associated with DSDs, either through a targeted sequencing panel or whole genome sequencing methods (Arboleda et al. 2013; Yang et al. 2013). Further, the NGS method offers a quick and unbiased approach for the detection of genetic mutations compared with the candidate gene approach. The latter can be expensive, slow, and may result in delayed diagnosis and initiation of appropriate treatment for cases of DSD. NGS is able to identify a genetic defect in almost 40% of DSD cases (Arboleda et al. 2014). It is important to note that different mutations/variations in the same gene can lead to a wide range of genital abnormalities (Baxter and Vilain 2013). Conversely, mutations in different genes can result in similar gonadal phenotypes. However, as the current understanding of pathogenic genetic variants in DSDs is limited, it is recommended to analyze the parental DNA along with that of the affected child where possible, with the purpose of narrowing down the pathogenic genetic variants, primarily in cases where a pathogenic mutation is not easy to identify (Di Resta et al. 2018). At the clinical level, efforts should be made to collect gonadal samples along with a peripheral blood sample from the individual, as the analysis of gonadal samples helps in revealing rare mosaicism, the nature of gonadal tissue, and any malignancy.

Whole genome or exome sequencing is not always the answer, and the exact approach for genetic diagnosis may vary depending on the setting, the resources available, and the ultimate aim of the investigation. Keeping in mind resource-limiting conditions in some countries and the availability of extensive genetic testing in others, we have drawn a simplistic view of the genetic testing approach in DSD cases (Fig. 1.2).

1.4.3 Fertility Potential and Counseling/Treatment Options

Fertility potential is significantly reduced in all types of DSDs. In a study of 1040 individuals with different DSDs, only 14% reported having at least 1 child, and only 7% had sought ART treatment (Słowikowska-Hilczer et al. 2017). In postpubertal males, the fertility potential can be sustained by sperm cryopreservation methods. In prepubertal males, spermatogonial stem cells (SSCs) can be cryopreserved, but at present, there is no way to differentiate them into spermatozoa for humans. There are some reports of achieving in vitro differentiation of spermatogonial stem cells into haploid cells in animals, but this has not yet been achieved for humans (Giudice et al. 2019; Ibtisham et al. 2020).

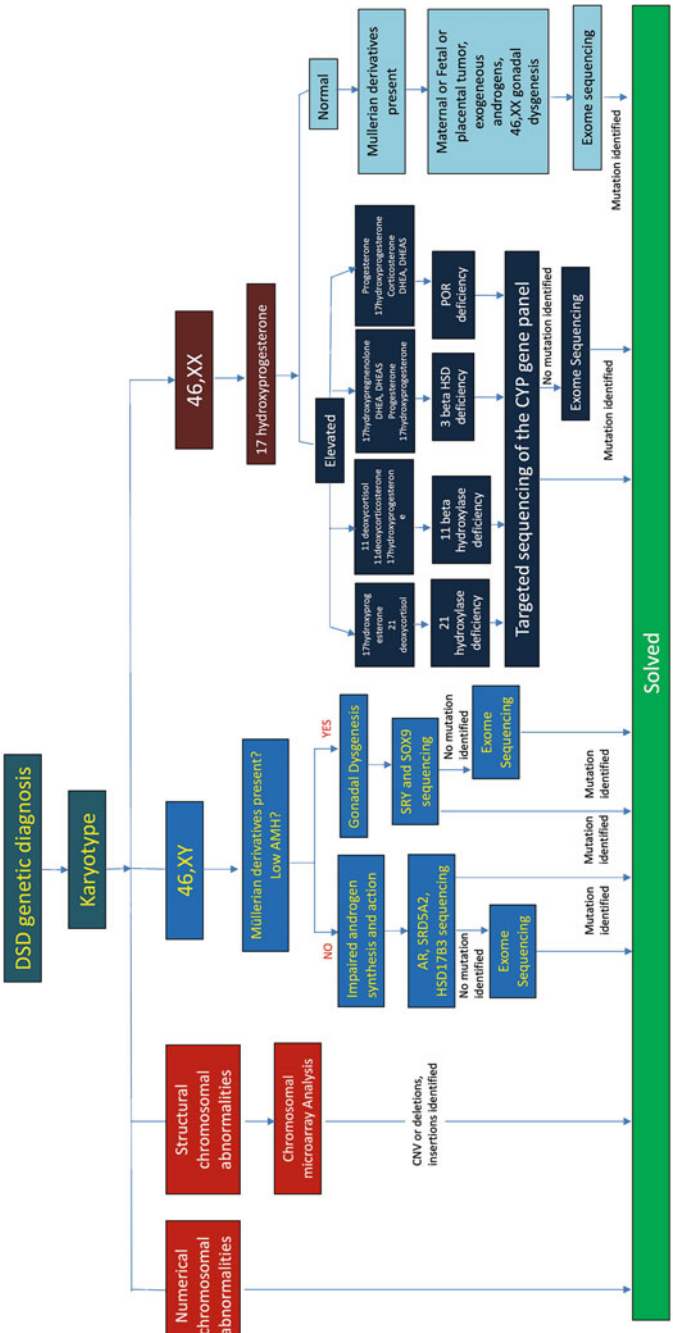


Fig. 1.2 Genetic testing approach for DSD cases

1.5 Conclusions and Future Perspective

Considerable progress has been made in the last few decades, and the technological advances have significantly improved the potential to identify pathogenic genetic mutations causing DSDs. Chromosomal analysis plays a central role in genetic analysis of disorders of sexual development (DSD), as a significant proportion of cases can be resolved solely by identifying chromosomal anomalies. In cases where no chromosomal anomaly is detected, additional analysis is typically pursued to identify genetic mutations and copy number variations, depending on the specific type of disorder. This multistep approach ensures a comprehensive evaluation of DSD cases, facilitating a more accurate diagnosis and appropriate management. In resource-limited conditions, a targeted sequencing approach focusing on specific genes relevant to the observed phenotype may be employed. This strategy allows for a more efficient use of available resources by focusing on the genes most likely to be associated with the observed clinical features. By selectively sequencing these genes, researchers and clinicians can gain valuable insights into the underlying genetic factors contributing to the phenotype, even with limited resources. For a thorough analysis, whole exome sequencing is now the preferred approach using specific panels of known pathogenic genes for the identification of mutations. Robust new technologies have accelerated the pace of genetic analysis; however, the exact explanation and validation of identified mutations as a cause of the observed phenotype may remain challenging in a number of cases. Furthermore, genetic alterations in a significant number of what may be initially considered unexplained DSD cases may never be established, and some phenocopies may be due to acquired rather than genetic causes.

The management of such cases has to be undertaken on the basis of phenotypic evaluation. For a number of patients with DSDs, fertility can never be achieved. In such a scenario, the restoration of the external genitals becomes the primary focus to enable the patients as adult to develop a functional sexual identity and gender role. In the case of affected children identified at an early age, surgery and hormonal treatment may be used to achieve the optimal morphological and psychological development corresponding to one or other of the binary of sexes. In other cases with delayed diagnosis during puberty or as adults, hormonal and surgical management are beyond the scope of this chapter. Nevertheless, for DSDs, genetic testing is an essential basis for firm diagnosis and optimal management.

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Y-Chromosome Deletion Testing in Infertility

2

Shruti Sethi and Rajender Singh

Abstract

Y-chromosome harbours genes critical for gonadal development and the maintenance of spermatogenesis. Deletions in the AZF region of the Y-chromosome lead to spermatogenic arrest, which can produce phenotypes ranging from mild oligozoospermia to azoospermia. Y-deletions account for approximately 14% of infertile individuals. Y-deletion analysis has been recommended by the European Academy of Andrology (EAA), and the European Molecular Genetics Quality Network (EMQN) in infertile males with sperm count below 5×10^6/mL. Y-chromosome deletions have a huge clinical significance in understanding infertility, choosing the method of treatment, and providing genetic counselling. In case of azoospermic individuals, Y-deletion analysis can help in predicting the presence of spermatozoa in the testis. Y-deletion analysis using sY84 and sY86 for AZFa, sY127 and sY134 for AZFb, and sY254 and sY255 for AZFc is sufficient. We recommend Y-deletion analysis in all infertile cases undergoing assisted reproduction.

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Keywords

Male infertility · Spermatogenesis · Oligozoospermia · Azoospermia · Genetic testing · Y-chromosome deletions · AZF deletions · Y-microdeletions · Reproductive medicine

2.1 Introduction

In mammals, the acrocentric Y-chromosome consists of pseudo-autosomal regions (PAR), along with a short arm (Yp) and a long arm (Yq). The two arms of the Y-chromosome are separated by the centromere. PAR1, which is 2.7 Mb in length, located at the tip of the short arm, is larger than the PAR2 region of 0.3 Mb, located at the apex of the long arm of the Y-chromosome. PAR1 and PAR2 contain a total of 29 genes (Otto et al. 2011; Colaco and Modi 2018). Genes present in the PAR1 region are majorly known for their role in growth and brain development. On the other hand, PAR2 contains only a few genes, such as IL9R, SPRY3, and SYBL1 (Jangravi et al. 2013). PARs undergo recombination almost similar to autosomes. Studies have shown that the Y-chromosome has a male-specific region, which is flanked by PARs at both the ends and harbours the major genes involved in male gonad development, spermatogenenic progression, and other organ development. On the Y-chromosome, euchromatin regions are located distal to PAR1 and consist of the short arm and a section of the long arm proximal to the centromere.

The short arm of the Y-chromosome hosts the SRY gene (sex-determining region on Y), which is crucial for sex determination. SRY was identified by way of deletion mapping (Sinclair et al. 1990). Along with the SRY gene, the AMELY (Amelogenin, Y linked), ZFY (zinc finger protein, Y linked), and TSPY genes also reside on the short arm of the Y-chromosome. Mutations in other genes present on the Y-chromosome have been found to be linked with various developmental disorders and male infertility (Rengaraj et al. 2015).

The long arm of the Y-chromosome harbours genes for spermatogenesis. Due to the palindromic structure of the Y-chromosome, recombination takes place in various combinations, leading to frequent inversions and deletions, often leading to various degrees of spermatogenic impairment. Based on the genetic analysis, a large stretch on the long arm of the Y-chromosomes, known as azoospermic factors (AZFs), has been found to be associated with male infertility. Genetic dissection of the AZF locus subdivided this region into three non-overlapping domains with the help of panels having various sequence tag sites (STSs): AZFa, AZFb, and AZFc. Yel3, Yel4, B5, B6, T1, and T2 are the only 7 of the 14 gene units that are unique to AZFb, whereas the remaining 10 are shared with AZFc (Fig. 2.1). In contrast to the above, the multiple copy locus of 12 genes, the Y-chromosome specific repeated DNA family (DYZ family), 14 amplicon units, and 19 repeat elements located in the AZFc locus make it a very complex structure (Ross et al. 2005).

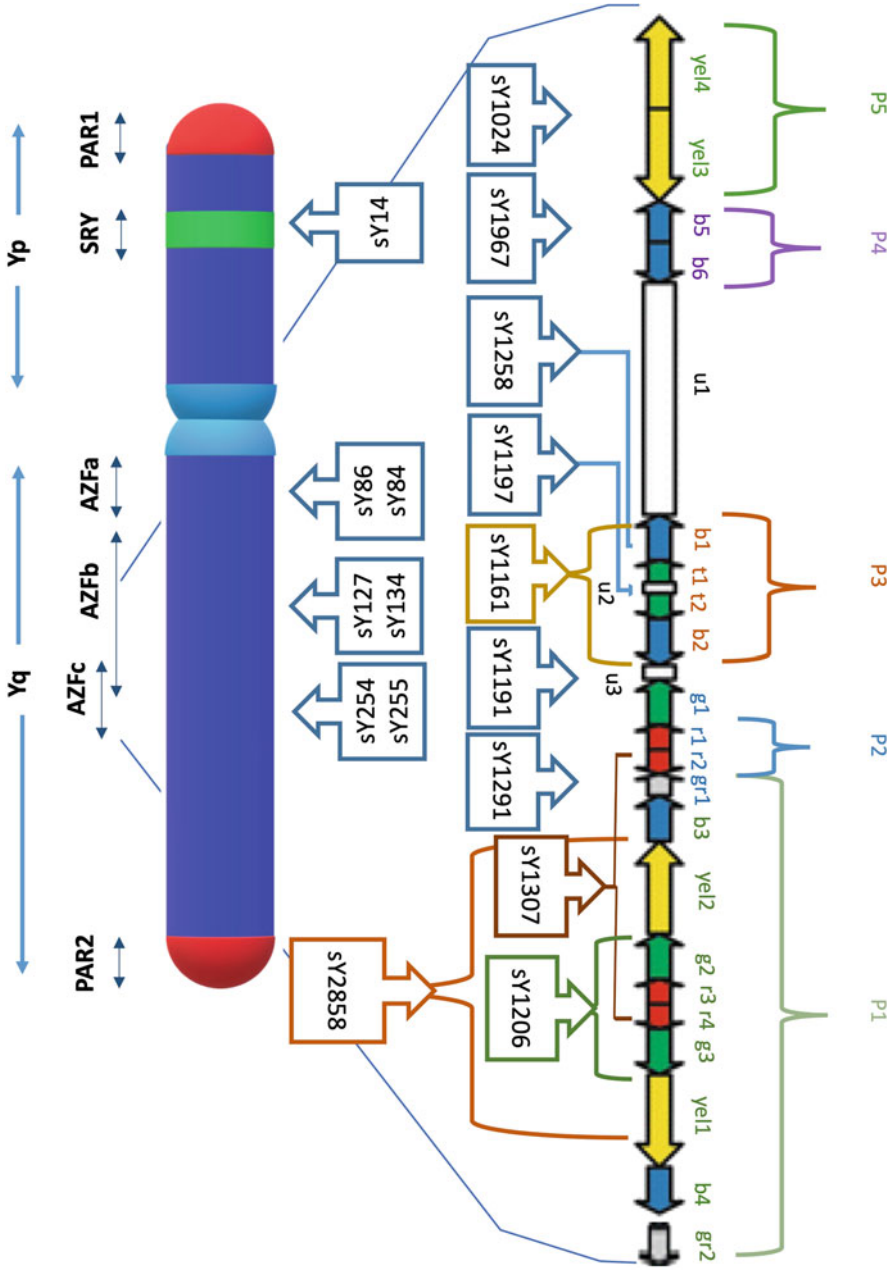


Fig. 2.1 Structure of the AZF region and the recommended STS markers for Y-chromosome deletions analysis

2.2 Y-Chromosome Microdeletions

Y-chromosome microdeletions were first correlated with spermatogenic failure by Tiepolo and Zuffardi (1976). Approximately 1 in 4000 men across all ethnic groups are infertile due to Y-chromosome deletions (Krausz et al. 2014). Azoospermia ($<1 \times 10^6$ sperm/mL semen) and mild ($10–15 \times 10^6$ sperm/mL semen) to severe oligozoospermia ($<5 \times 10^6$ sperm/mL semen) conditions have been found in association with defects on the Y-chromosome (Krausz et al. 2018). Thorough investigations have shown that about 5% of the cases of severe oligozoospermia and 13% of azoospermia are caused by Y-chromosome deletions (Bansal et al. 2016). Since spermatogenesis is directly dependent on the Y-chromosome genes, investigation of Y-deletions has been suggested to be clinically important (Sen et al. 2013).

Mostly, deletions on the long arm of the Y-chromosome have been studied and found to be strongly associated with male infertility. SRY deletion on the short arm generally causes defects in sex determination (Vetro et al. 2015). Deletions of other genes located on the short arm have not been directly linked to infertility but confer a high risk of various developmental disorders; such as deletion of PCDH11 can cause speech defects in the carrier male (Speevak and Farrell 2011).

Globally, the incidence of AZF deletions in infertile men has been reported to be 7% (Colaco and Modi 2018). Widely studied deletions in AZFs on the long arm have been found to be strongly associated with oligozoospermia and azoospermia conditions (Rani et al. 2019). Yq microdeletions have been detected 15–20% more often in azoospermic men compared to oligozoospermic cases (Krausz et al. 2014; Romo-Yáñez et al. 2022). About 25–55% of males with various testicular abnormalities have been diagnosed with Y-chromosome microdeletions (Bansal et al. 2016). Deletions on the Y-chromosome were first identified in the AZFc region, with an 80% prevalence, followed by deletions in the AZFa (0.5–4%), AZFb (1–5%), and AZFbc (1–3%) regions (reviewed by Krausz et al. 2014). Recent studies have also confirmed the association of dysregulated hormonal level along with decreased sperm count in AZFs deletion carrier males (Lahoz Alonso et al. 2023). In India, the incidence of AZFc deletions has been reported to be lower than in Western countries, but the incidence of AZFa deletions is higher in Indian populations (Waseem et al. 2020). These deletions have been clinically categorized into (1) classical or complete deletions and (2) partial deletions based on the length of the deletion in the AZF region.

2.2.1 Classical Deletions

These deletions encompass large regions of the long arm of the Y-chromosome, often covering multiple regions, including AZFa, AZFb, and AZFc regions in various combinations (Repping et al. 2003). Due to the involvement of large regions, these deletions often lead to complete failure of spermatogenesis (Ferlin et al. 2007). Reports have shown that complete deletion of AZFb+AZFc can cause

hypospermatogenesis, which is due to the deletion of genes, leading to spermatogenic arrest at the maturation phase of spermatozoa (Navarro-Costa et al. 2010). Complete deletion of a 6.2 Mb stretch (consisting of about 32 genes) during homologues recombination of P5/P1 palindromic sequences has been reported in infertile males (Soares et al. 2012). Complete deletions of AZFa can lead to Sertoli-cell syndrome or azoospermia, as they cause the complete loss of germ cells. Microdeletions of the AZFb have been detected in 1–5% of cases of Yq deletions and often lead to complete loss of spermatogenesis (Hinch et al. 2014). Similarly, the deletion of the complete AZFc region leads to azoospermia (Repping et al. 2002). An example of complete deletion in AZFc is the deletion of the b2/b4 region. In azoospermic males, Ma et al. (1993) identified deletions in the AZFc (50f2/C) region (Ma et al. 1993). Several case studies have also reported a link between the classical AZFc deletions and severe oligozoospermia and azoospermia (Yao et al. 2001). Although the number of spermatozoa in the ejaculate of AZFc deletion carrier males is low, they can still go for cryopreservation. Moreover, even after a drastic decrease in sperm count, a few cases of AZFc deletions remained fertile (Stouffs et al. 2011).

2.2.2 Partial Deletions

Partial deletions are defined as the deletion of some part of the AZFa, AZFb, or AZFc regions. As expected, partial deletions generally have a milder impact on spermatogenesis but may result in infertility. Although gene-specific deletions in the Yq region are not commonly reported, AZFa partial deletions have been found to result in deletions of critical genes, such as USP9Y, which showed phenotypes ranging from azoospermia to normozoospermia (Luddi et al. 2009; Kleiman et al. 2012).

Partial deletions in the AZFb region are rare and less impactful as compared to complete deletions due to the retention of a few copies of genes such as HSFY, CDY2, and XKRY (Stahl et al. 2012). Another study detected a 768 kb deleted region in four infertile men covering the P4 region, which consisted of the HSFY1 and HSFY2 genes along with several other genes (Krausz et al. 2014), confirming the link between partial deletions of AZFb and male infertility.

AZFc does not contain single copy genes, but the repetitive and palindromic nature of this region makes it highly prone to rearrangements and deletions, leading to reduced sperm count (Kuroda-Kawaguchi et al. 2001; Vogt 2004). Partial deletions reported in the AZFc region are b1/b3 (1.6 Mb), gr/gr (1.6 Mb), and b2/b3 (1.8 Mb) (Repping et al. 2003). Most of the deletions are known to eliminate a few copies of various gene families located in this region (Repping et al. 2003). Gr/gr deletion tends to eliminate two copies of the DAZ gene along with a few copies of the CDY and BPY2 genes, resulting in mild oligozoospermia to azoospermia, which more or less depends on the geographic origin and ethnic group of patients (Saut et al. 2000). Gr/gr deletion is seen in 10–15% of infertile cases in Asia and Africa, which is higher than other populations (Bansal et al. 2016). Additionally, it has also been proposed that the gr/gr deletions expand and eventually become

complete deletions (Ferlin et al. 1999). Moreover, gr/gr deletion carriers are found to be at a higher risk of testicular germ cell tumour (Stouffs et al. 2011; Bansal et al. 2016). About 50% of azoospermic men with AZFc deletions have chances of spermatozoal retrieval with TESE (Ferlin et al. 2007; Simoni et al. 2008).

Ten percent of cases of defects in spermatogenesis have been found to have deletions in the DAZ gene located in the AZFc region (Kuo et al. 2004). Another type of deletion, b1/b3, was observed in 1 in 994 men during the analysis of 20,000 Y-chromosomes (Rozen et al. 2012). This deletion has been found to be deleterious as it leads to the elimination of two copies of PRY and the deletion of six copies of RBMY1 (Rozen et al. 2012). Recently, a study investigated the effect of miRNAs targeting genes deleted in AZF, which may bind to the competing endogenous mRNA and contribute to infertility in AZF deletion carriers (Ergun et al. 2022).

2.3 Why Screen for Y-Deletions?

2.3.1 The European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) Recommend Y-Deletion Analysis

The European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) provided guidelines for screening Y-chromosome deletions in males with sperm counts <1–5 million/mL and <5–20 million/ml to undergo genetic testing (Krausz et al. 2014). Some later studies suggested this cutoff is not suitable as the average sperm count has decreased globally (Sengupta et al. 2017). Recent studies have recommended a genetic diagnosis below 1×10^6 sperm/mL (Kohn et al. 2019). We advocate genetic analysis for Y-deletions in all cases undergoing assisted reproduction due to male factor infertility, irrespective of sperm count. The major reason for this suggestion is that apart from helping predict the chances of success in assisted reproduction, Y-deletion analysis becomes critical for patient counselling. Y-deletions account for an average of 10% of male factor infertility cases, which is a very high percentage. Even if these patients can be treated without Y-deletion analysis, they never realize the risk of transmitting these deletions to their offspring. Therefore, we advocate Y-deletion testing in all cases seeking assisted reproduction due to male factor infertility.

2.3.2 Y-Deletion Screening Helps in Selecting the Method of Treatment

After the arrival of an infertile patient at the clinic, there are various methods of treatment that can be instituted. Though IVF and ICSI promise fatherhood to a very high percentage of infertile individuals, these procedures involve various ways of sperm retrieval, the use of donor sperm, and the risk of the failure of a cycle, which call for a cumulative accounting before deciding the adoption of a particular method.

In a significant number of male factor infertility cases, treatment using hormonal therapy, antioxidants, vitamins, or other means may be advised. Since Y-deletions remove genes critical to spermatogenesis, Y-deletion analysis in such cases can help predict the chances of success. In cases where the patient is open to any method of treatment, the analysis of Y-deletions on the first visit to the clinic can help identify Y-deletions and subsequently choose a method of treatment depending on the presence/absence of Y-deletions. An individual with Y-deletions is less likely to respond to hormonal or antioxidant treatments and should be advised to undergo assisted reproduction in the first instance for better success. In a recent study on the impact of Y-deletions on the success of hormonal therapy, we observed that individuals with Y-deletions were less likely to respond to the hCG therapy aimed at improving semen parameters (Andrabi et al. 2022). Irrespective of whether a person was advised to undergo assisted reproduction upon detection of Y-deletion or upon failure of hormonal/antioxidant treatment, counselling can be offered to the patient regarding the transmission of the Y-deletions to the offspring. In such a case, the patient may consider going for donor sperm, but irrespective of his decision, he will be aware of the risk of transmitting Y-deletions to his offspring.

In the case of azoospermic patients, sperm retrieval from the testis is a commonly used method of sperm retrieval. This technique, called testicular sperm extraction (TESE) or its in-depth version, named micro-TESE, promises fatherhood to about 30–70% of azoospermic individuals. While the technique helps the patients, it is a painful procedure with a very high chance of failure to locate spermatozoa in the testis. Y-deletion analysis comes in handy in such cases. The detection of complete AZFa and AZFb deletions often results in complete azoospermia with almost 'nil' chance of finding sperm in the testis; therefore, such patients should be advised to go for donor sperm without TESE or ICSI. On the other hand, individuals with AZFc have a high chance of finding spermatozoa upon TESE. These individuals should be advised to undergo TESE or micro-TESE with the hope of finding at least one sperm for ICSI (Fig. 2.2).

Y-deletions also render individuals susceptible to faster deterioration of sperm count over a period of time (Bansal et al. 2016). Therefore, the presence of Y-deletions may result in further deterioration of sperm count, rendering it more difficult to undertake assisted reproduction in the future. We advise the patients to undergo Y-deletion analysis as soon as possible in order to get help by selecting the best-suited method of treatment.

2.4 Methods for Screening Y-Deletions

Methods available for screening Y-chromosome deletions include multiplex PCR and array comparative genomic hybridization (aCGH). However, technological advancements have prompted the use of NGS-based methods to detect Y-chromosome deletions. Multiplex PCR is taken as the gold standard technique that uses sequenced tagged sites (STS) primers to amplify and detect Y-chromosome deletions in the AZFa, AZFb, and/or AZFc regions (Repping et al. 2003;

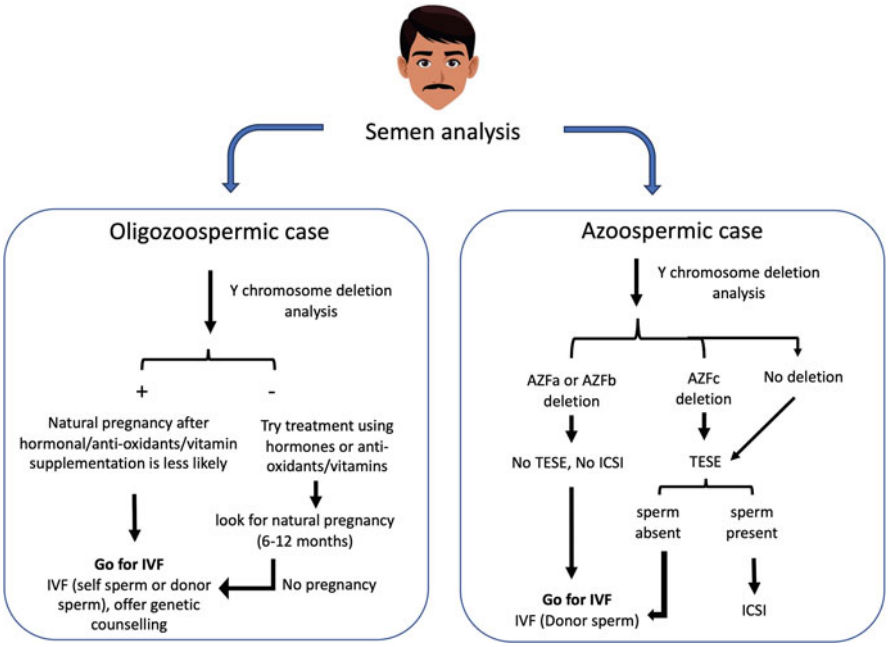


Fig. 2.2 A schematic guide chart for using Y-deletion analysis in infertility treatment

Thirumavalavan et al. 2019). The analysis of two STS loci in each region increases diagnostic accuracy, even though the analysis of a single non-polymorphic STS is potentially adequate (Liu et al. 2016). Recommended primers for detecting deletions are sY84 and sY86 for AZFa; sY127 and sY134 for AZFb; and sY254 and sY255 for AZFc regions (Krausz et al. 2014) (Fig. 2.1). EAA and EMQN recommended using internal positive and negative controls for multiplex reactions for testing. An inconclusive diagnosis or error in testing should be confirmed by repeat testing (Krausz et al. 2014).

Array comparative genomic hybridization (aCGH) is another technique commonly used to detect copy number variations using microarrays (Thirumavalavan et al. 2019). Studies have proven that high-resolution aCGH can identify even those CNVs that are missed by conventional PCR techniques. However, inaccuracy in detecting partial duplications and a high cost per patient are major limitations of aCGH (Yuen et al. 2014).

Recent advancements in sequencing techniques, such as NGS-based sequencing, are now being used to diagnose Y-chromosome deletions (Patel et al. 2018). Several panels have been developed to detect single gene polymorphisms, CNVs, and other chromosomal abnormalities with high accuracy, but Y-chromosome microdeletion analysis through NGS-based techniques is not common as conventional short read sequencing by NGS provides only 94% accuracy in detecting Y-chromosome deletions (Thirumavalavan et al. 2019). Nevertheless, the emerging technology of

NGS promises to offer the detection of some unknown and incomplete Y-chromosome deletions. Recently, the human Y chromosome has been fully sequenced using Oxford Nanopore ultralong reads and PacBio high-fidelity reads, in addition to enhanced assembly algorithms which produced a thorough genetic blueprint of human genetics (Rhie et al. 2023). Moreover, studies have discovered the advantage of using NGS in the diagnosis of Y-chromosome deletion compared to STS-PCR, because missed deletions were detected by the NGS-based approach, which PCR was not able to detect (Liu et al. 2021). As discussed above, the extent of deletion in any domain of the Y-chromosome strongly correlates with the phenotype of infertility, as complete deletions can cause a more severe phenotype and reduce the chances of sperm retrieval with TESE for ICSI. Therefore, the extent of deletions in a region of the Y-chromosome should also be considered for predicting the success of ART and infertility treatment.

2.5 Type of Sample for Y-Deletion Testing

Earlier, it was thought that undertaking Y-deletion testing in peripheral blood samples was enough. However, germline deletions have been found in a number of cases, where peripheral blood analysis failed to identify them. It may be due to their germline origin in these cases. Further, there could be longer deletions in the germ cells in comparison to the peripheral blood in some cases, making it important to analyse the germ line for Y-deletions (Dada et al. 2007; Sakthivel and Swaminathan 2008). Moreover, recent research has investigated the AZF deletions in germ cell genomic DNA and suggested the use of sperm DNA to check the status of the genomic landscape in infertile men (Archana et al. 2023). Therefore, ideally, sperm DNA should be used for Y-deletion testing, and since the individual has to undergo semen analysis as a part of the investigation, the same sample can serve both purposes and also reduce the burden of handling a blood sample.

2.6 Y-Deletion Testing Is Required for Offering Genetic Counselling

Since Y-deletions are seen in more than 10% of male infertility cases, genetic counselling for these patients requires knowledge of their Y-deletion status. Irrespective of the method of treatment opted for, these individuals would pass their Y-deletions to the next generation, raising infertility risk in their offspring as well. Therefore, the detection of Y-deletions is generally used to provide genetic counselling to these individuals regarding the vertical transmission of the deletions to the offspring (Silber and Repping 2002). As Y-chromosome deletions are not only associated with male infertility but also increase the risk of several other developmental disorders and pathological conditions, such as testicular cancer, their analysis can help in the pre-diagnosis of some serious health conditions (Bansal et al. 2016; Castro et al. 2017). An early diagnosis in mild oligozoospermia cases can provide a

complete status of the genetic landscape of the Y-chromosome, which can aid in genetic counselling and cryopreservation of spermatozoa in these cases. In some cases, infertile patients may prefer to go for donor sperm, which can completely eliminate the vertical transmission of Y-deletions.

2.7 Conclusions and Future Perspective

Y-chromosome deletions hamper various crucial processes such as gonadal development, initiation and maintenance of spermatogenesis, and they are considered to be the second most frequently diagnosed genetic cause of male infertility. Recent technological developments have made it possible to detect Y-chromosome deletions with a great deal of accuracy and forecast the possibility of the effectiveness of hormonal therapy in treating male infertility. It has been demonstrated that Y-chromosome deletion identification can help in genetic counselling and to assess the success of ART. Additionally, Y deletions show a link with genomic instability and the chance of developing various clinical disorders that can be brought on by Y-chromosome abnormalities. A careful examination of Y-chromosome deletions can show promising results in resolving the unexplained factors of infertility and provide valuable insights in the diagnosis and treatment of male infertility. Next-generation sequencing may bring paradigm shift in screening Y-deletions in the future.

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Genetic Testing in Male Infertility

3

Shruti Sethi and Rajender Singh

Abstract

The problem of infertility is rising rapidly. Male fertility is greatly influenced by genetic factors, with chromosomal abnormalities and single-nucleotide polymorphisms (SNPs) having a significant impact in complex with the environment. The genetic testing in male infertility is important for understanding the genetic causes and to offer genetic counseling to patients. Chromosomal abnormalities can now be diagnosed more easily by karyotyping, FISH, and array comparative genomic hybridization (aCGH). In addition to Y-chromosome deletions and chromosomal aneuploidies, SNPs within genes critical to spermatogenesis and hormone regulation can also affect male fertility. The risk of male infertility has been associated with genetic polymorphisms in follicle-stimulating hormone receptor (FSHR), androgen receptor (AR), cystic fibrosis transmembrane conductance regulator (CFTR), deleted in azoospermia like (DAZL), DNA polymerase subunit gamma (POLG), estrogen receptor (ESR), and methylenetetrahydrofolate reductase (MTHFR) genes. However, polymorphisms in all of these genes are not consistently associated with male infertility. In this chapter, we present the autosomal gene, polymorphisms which make a good case for testing in male infertility.

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Male infertility · Genetics of male infertility · Genetic testing · Genetics of oligozoospermia · Genetics of azoospermia · Reproductive medicine · Genetic polymorphism

3.1 Introduction

Investigating the genetics of male infertility is quite complicated, as around 4000 genes participate in a highly coordinated pattern to accomplish the production of fertile spermatozoa. Male factors contribute to up to 50% of infertility cases (Kumar and Singh 2015). Our previous study on semen profile across a period of 37 years revealed a significant decline in semen quality in Indian men (Mishra et al. 2018). Similar trends have been reported from other parts of the world, including North America, Europe, and Australia (Levine et al. 2017). Approximately, 20–30% of infertile men show oligozoospermia or azoospermia. Research aimed at understanding the causes of infertility has advanced our understanding of infertility. We believe that genetic abnormalities may account for about 40% of cases of infertility, whereas the other 40% may be due to the environment, and in the rest 20% of the cases, a complex interaction between genetics and the environment underlies infertility. Nevertheless, infertility in about 80% of individuals remains idiopathic.

About 10% of female and 15% of male infertile cases are associated with various chromosomal anomalies and single-nucleotide polymorphisms (Foresta et al. 2002). Men with <5 million sperm count/mL along with a higher FSH level or lower testosterone and LH levels are highly advised to undergo genetic testing prior to infertility treatment (reviewed by Wosnitzer 2014). In order to study the genetics of male infertility, the American Urologic Association (AUA), the European Association of Urology (EAU), and the American Society of Reproductive Medicine (ASRM) have recommended an initial workup on infertile patients that involves a detailed study of medical history, physical examination of external genitalia, and the analysis of various semen parameters before going for genetic testing (Jungwirth et al. 2016). Karyotyping, CFTR, Y-deletion, or other genetic tests are recommended depending on the clinical history of the patient. One of the benefits of advancement in sequencing technology is comparative genomic hybridization arrays, which identify uncommon variations in hundreds of genes that have been explored as prospective candidates for male infertility. A higher risk of transmission of genetic abnormalities to the offspring is also a concern. In this chapter, we will briefly discuss the probable genetic anomalies that should be considered during the genetic diagnosis and treatment of male infertility.

3.2 Various Types of Genetic Abnormalities

Both numerical and structural chromosomal anomalies have been found to be associated with oligozoospemia and azoospermia, ranging from 1.10% to 13.33%, and 10% to 23.62%, respectively (Ceylan et al. 2009). Moreover, several chromosomal defects have been identified in 10–15% of infertile males with the nonobstructive azoospermia phenotype (Yoshida et al. 1997; Arafa et al. 2017).

3.2.1 Numerical Chromosomal Abnormalities

Karyotyping of infertile men identified numerical or structural abnormalities in 2%–14% of individuals (Foresta et al. 2005). This includes the 47,XXY karyotype (Klinefelter syndrome) in 11% of azoospermic and 0.7% of oligozoospermic men (reviewed in De Braekeleer and Dao 1991). The first evidence of 47,XXY was found in 1942 (Jacobs and Strong 1959); currently, the prevalence of this syndrome is 4 patients per 10,000 with a frequency of 0.04% males in the general population (Bojesen et al. 2003). Recent advances in ART, such as testicular sperm extraction (TESE) and specialized microsurgical methods, have ensured higher success rates of 30–70% for spermatozoa retrieval during ICSI in males with the 47, XXY karyotype (Seo et al. 2004; Schiff et al. 2005). 48,XXYY and 49,XXXXY karyotypes are other examples of the consequences of chromosomal abnormal segregation, which are diagnosed in 1 in 17,000 to 50,000 newborn males and 1 in 85,000 to 1,00,000 male births, respectively (Linden et al. 1995). Mixed gonadal dysgenesis, or 45X/46XY mosaicism, is a rare condition with an incidence of 1 per 15,000 newborns (Lindhardt Johansen et al. 2012). Individuals with more than 35 years of age or a family history of genetic abnormalities are at a higher risk of giving birth to a child with an abnormal karyotype. Guidelines have been provided by an international consortium of experts of the EAU, recommending a karyotype analysis for men having a sperm count of less than 10 million/mL (Jungwirth et al. 2016), whereas the AUA recommends karyotyping in men with a sperm count of less than 5 million/mL (AUA 2006).

3.2.2 Structural Chromosomal Abnormalities

This includes complete and partial chromosomal deletions, inversions, and translocations of genetic material between chromosomes. Chromosomal deletions were first reported on the Y chromosome, which occur more commonly in the AZFc region with a frequency of 80%, followed by deletions in the AZFa, AZFb, and AZFbc regions at frequencies of 0.5–4%, 1–5%, and 1–3%, respectively (Waseem et al. 2020). The classical deletions (large) were identified in 80% of all AZF deletion events, followed by microdeletions, which accounted for infertility in 3–13% of individuals (Waseem et al. 2020). The detection of partial deletions was the last entry in the group of Y-deletions, which account for infertility in 20% of men with low

sperm counts (Krausz et al. 2000). Men with severe oligozoospermia (2–5%) and nonobstructive azoospermia (NOA) (5–10%) are more likely to have Y-chromosome microdeletions (Foresta et al. 1998). Deletions of the AZFa region lead to a complete absence of spermatozoa, resulting in the development of Sertoli cell only syndrome (Waseem et al. 2020). Furthermore, complete deletions of the AZFb+AZFc region have also been reported in association with male infertility. A detailed account of Y-deletions and their testing has been provided in Chap. 2.

A range of 0.9%–3.5% infertile men having severe spermatogenic abnormalities have been found to be associated with Robertsonian translocations with 5–32% of sperm with an unbalanced translocation present in the ejaculate of a Robertsonian translocation carrier individual (Therman et al. 1989; Frydman et al. 2001; Pylyp et al. 2013). A few reciprocal translocation cases investigated with the help of karyotyping and FISH have been found to be linked with a lower survival rate along with higher mental and physical impairments in newborns (Boué and Gallano 1984). Another uncommon chromosomal anomaly, 46,XX, often known as de la Chapelle syndrome, was first identified in 2001 and affects 1 in 20,000–30,000 males (de la Chapelle 1972). About 90% of the cases with SRY-positive XX syndrome may show normal external genitalia or genital ambiguity in some cases, along with testicular biopsy showing no evidence of abnormal spermatogenesis (Zenteno et al. 1997; Vorona et al. 2007). The rest 10% of the cases have been found to be SRY- negative 46, XX syndrome. A strong correlation has been detected between the frequency of chromosomal abnormalities in the fetus and the reciprocal translocation carrier father (Escudero et al. 2003). We detected duplication in 19p13.3 in different cases of infertility and reported a higher frequency of this duplication in SCOS (38%) cases, which underlines its potential as a unique biomarker to diagnose male infertility (Singh et al. 2019). 46, XY, inv. (17) (p13.1q25.3), a pericentric chromosome 17 inversion, was reported to have 73% of sperm with balanced translocation (Mikhaail-Philips et al. 2005). Another study reported 50% of unbalanced and 50% of balanced translocation carrying sperm after analysing 10,007 spermatozoa produced in inv. (6) (p23q25) carrier individuals (Anton et al. 2002).

3.2.3 Single-Nucleotide Polymorphisms

While the Y-chromosome harbors the principal genes for spermatogenesis, other genes on the X-chromosome and autosomes also contribute to spermatogenesis directly or indirectly. As a result, research in the genetics of male infertility has widened its scope to investigate the role of SNPs, VNTRs, and Y-chromosome-linked CNVs. Some autosomal polymorphisms have been investigated in more than one population. Some of the SNPs have been very well studied, including meta-analyses assessing their impacts (Tüttelmann et al. 2011; Ghadirkhomi et al. 2021). The collection of these mutations can be exploited to design genetic panels for screening for the risk of infertility. On the basis of genetic understanding of infertility, we have suggested a workflow for genetic analysis of infertile individuals (Fig. 3.1).

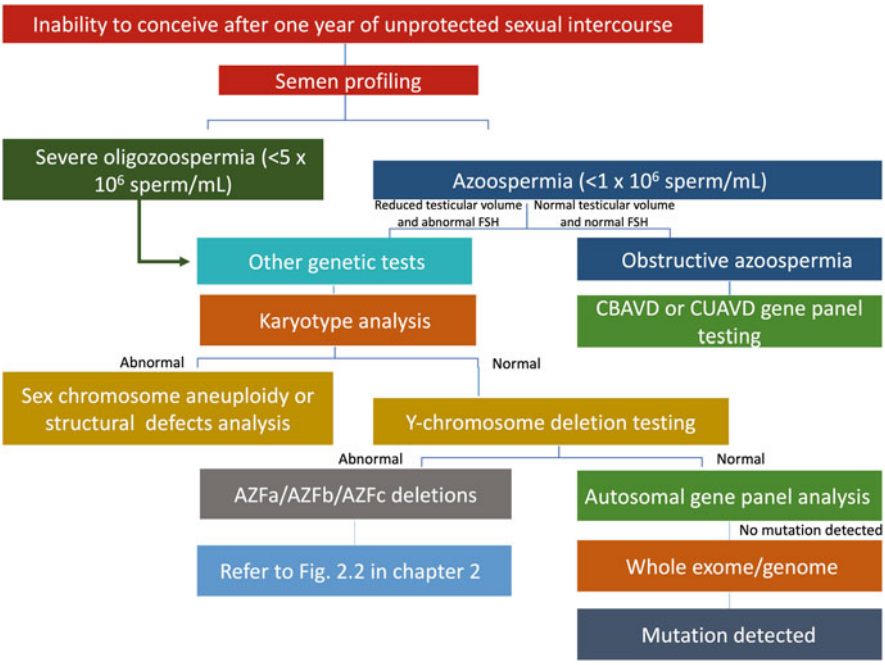


Fig. 3.1 Flow chart to guide genetic testing in infertile patients

3.2.3.1 Androgen Receptor (AR) Gene

AR is critical to male development and fertility. Hundreds of mutations in AR have been reported to cause infertility. Approximately 2% of infertile males are affected by the aberrant androgen-binding activity of AR (Vockel et al. 2021). The most severe forms can be identified by the abnormal development of the external genitalia. About 1064 mutations mapping to almost all exons of the AR gene have been reported to result in an array of phenotypes ranging from ambiguous genitalia in newborns to "idiopathic" male infertility, depending on the extent of the loss of AR function (Gottlieb et al. 2012). Males with abnormal external genitals (partial androgen insensitivity syndrome) or those with under-masculinization (mild androgen insensitivity syndrome) or oligozoospermia should be analyzed for AR mutations. Rapid investigations revealed novel mutations associated with different subtypes of male infertility, such as a significant linkage of Gly684Ala substitution (c.2051G > C) with azoospermia in the Chinese population (Yuan et al. 2022). AR needs complete sequencing of the coding region, as mutations across the whole coding region have been reported to cause male infertility (Yong et al. 2000).

3.2.3.2 Follicle-Stimulating Hormone Receptor (FSHR) Gene

Follicle-stimulating hormone (FSH) binds to its membrane-bound receptor (FSHR) on Sertoli cells and indirectly supports the maturation of viable spermatozoa (Oduwole et al. 2018). A few SNPs in the FSHR gene have been investigated and

suggested to be great candidates for screening in male infertility, for example, G/A substitution in the core promotor (G29A, rs1394205) and other common SNPs causing substitution of threonine by alanine at codon 307 (919A/G, Thr307Ala, rs6165) and substitution of asparagine by serine (2039A/G, Asn680Ser, rs6166) (Li et al. 2011). An assessment of the significance of all possible haplotypes constituted by the three SNPs in the FSHR gene revealed a high frequency of the G-29-A919-A2039 haplotype in normozoospermic males when compared with azoospermia patients, hence showing the protective nature of this haplotype in males (Gharesi-Fard et al. 2015). However, a previous meta-analysis on FSHR Thr307Ala and Asn680Ser polymorphisms did not show any significant association with male infertility (Wu et al. 2015), which demands more studies be done to draw a conclusion about the significance of these SNPs in attesting the risk of male infertility.

3.2.3.3 Glutathione S-Transferase (GST) Gene

Glutathione S-transferase belongs to a major defensive superfamily of proteins that act against oxidative stress, which is well studied in the context of sperm DNA damage and male infertility. In humans, the GSTA, GSTM, GSTK, GSTO, GSTP, GSTS, GSTT, and GSTZ genes encode eight classes of GSTs- α (alpha), μ (mu), κ (kappa), ω (omega), π (pi), σ (sigma), θ (theta), and ζ (zeta), respectively (Liang et al. 2004). Gene polymorphisms in members of the GST gene superfamily have been shown to be associated with male infertility (Safarinejad et al. 2010). The presence of mutations in the GSTM1, GSTT1, and GSTP1 genes affects the binding affinity of the enzymes encoded by these genes, which can cause nonfunctional or less functional enzymes, leading to oxidative stress, DNA damage, and male infertility (Liang et al. 2004). Studies have shown the impact of null mutations in the GSTM1 and GSTT1genes on the increased risk of male infertility (Safarinejad et al. 2010; Tirumala Vani et al. 2010). The conclusion drawn by a meta-analysis also supports a very strong and significant association of GSTT1 and GSTM1 null polymorphisms with male infertility (Wu et al. 2012).

Genetic variant in exon 5 of the GSTP1 gene leads to the exchange of Ile with Val at the 105 position (rs1695) and influence the binding affinity and activity of this GST enzyme (Yadav et al. 2020). Decreased activity of GST enzyme leads to the it's inability to detoxify mutagens and protect DNA (Liang et al. 2004). In idiopathic male infertility, the sperm DNA fragmentation index was found to be increased in patients with the GSTT1 (−) and M1/T1 (−/−) genotypes (Zhang et al. 2021). Men with GSTP1 C/T are at threefold and 2.5-fold higher risk of having sperm with abnormal motility and morphological abnormalities, respectively (Myandina et al. 2019).

3.2.3.4 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene Mutations Leading to CBAVD Risk

The CFTR protein is an ion channel that helps regulate the efficient transport of anions and fluid excretions in the body. Eighty percent of infertile men with one or more mutations in the CFTR gene are known to suffer from obstructive azoospermia

caused by congenital bilateral absence of the vas deferens (CBAVD) (Patat et al. 2016). The anatomical features of CBAVD include atrophy of the seminal vesicle, failure in bilateral vas deferens formation, and the lack of a significant portion of the epididymis (Patrizio and Zielenski 1996). Disorders like cystic fibrosis and other CF-related conditions like CBAVD are difficult to diagnose due to the lack of diagnostic facilities and insufficient knowledge and awareness of vas aplasia among general practitioners. The current ASRM, ACOG, and ACMG guidelines advocate the genetic evaluation and risk forecasting of CBAVD using enhanced CFTR mutation testing or carrier screening of the 23 most common (hot spot) CFTR variants (Elliott et al. 2012). In CBAVD patients, several CFTR mutations have been investigated. Interestingly, these mutations are not homogenous and show great variation in spectrum and frequency (Claustres et al. 2000; Dayangaç et al. 2004; Bombieri et al. 2011). The three most common mutations that lead to CBAVD are F508del, IVS8-5T (c.1210-12T), and R117H (c.350G. A) (Yu et al. 2011). In Caucasian populations, Kumar et al. (2014) reported the frequency of the p. F508del mutation ranging from 35 to 74.6% in CBAVD patients using the ARMS-PCR technique (Kumar et al. 2014). Similarly, the overall frequency of the occurrence of a CFTR mutant allele in the Taiwanese population with CBAVD is 36%, and five significant mutations have been found in Taiwanese men, p.V201M, p.N287K, c.–8G > C (125G > C), p.M469I, and p.S895N (Lin and Lin 2010). Further research needs to be undertaken to identify population-specific mutation panels for CBAVD disorder in various ethnic populations. Moreover, population-based studies can also help us identify unique as well as common causes of CBAVD across various populations (Sharma et al. 2009). Meta-analyses of studies on CFTR gene polymorphisms showed a significantly higher risk of male infertility associated with the IVS8-5T variant in non-European males as compared to European males (Levkova et al. 2022). Moreover, meta-analysis showed a higher risk of spermatogenic disruption in homozygous 5T/5T variant carriers compared to other IVS8-T variants. However F508del did not show a significant association with infertility (Yang et al. 2020).

3.2.3.5 Methylenetetrahydrofolate Reductase (MTHFR) Gene

The MTHFR gene is responsible for DNA methylation, which regulates the physiology of cells via gene silencing (Gong et al. 2015). Studies done so far have investigated the potential of MTHFR gene polymorphisms as an important marker for male infertility (Gupta et al. 2011). While more than 20 SNPs have been reported in the MTHFR gene, 677C > T and 1298A > C polymorphisms have been closely related to male infertility in a number of populations (Stuppia et al. 2003; Dhillon et al. 2007; Singh et al. 2010). MTHFR 677 C > T polymorphism has been studied in relation to male infertility in Asian (Safarinejad et al. 2011), European (Gurkan et al. 2015), and Caucasian populations (Nikzad et al. 2015). A meta-analysis from our lab on 29 studies identified that the MTHFR 677 C > T polymorphism is a significant risk factor for male infertility and should be analyzed in clinical settings (Gupta et al. 2011).

Epigenetic changes have also been studied in testicular biopsies of obstructive azoospermic males. Hypermethylation of the MTHFR promoter in NOA patients suggested that azoospermic infertility may be caused by epigenetic silencing of MTHFR in nonobstructive azoospermia (Khazamipour et al. 2009). Aliakbari et al. (2020) undertook subgroup analyses of MTHFR polymorphisms based on ethnicity; the results showed substantial correlation in Asians and Caucasians for 677C/T (rs1801133) and in just Asians for 1298A/C (rs1801131) (Aliakbari et al. 2020). In conclusion, MTHFR 677C/T seems to be a potential candidate in the diagnosis of male infertility, whereas MTHFR 1298A > C in relation to infertility demands further research based on population origin.

3.2.3.6 Deleted in Azoospermia-Like (DAZL) Gene

The DAZL gene, located on chromosome 3, is an autosomal homologue of the DAZ gene on the Y-chromosome, and it is necessary for germline fate determination (Ruggiu et al. 2000). DAZL gene polymorphisms (260A > G and 237A > G) have been shown to increase the risk of male infertility in Indian populations (Thangaraj et al. 2006). Another DAZL (T54A) polymorphism has been reported to increase the probability of development of oligozoospermia and azoospermia in males (Shafae et al. 2018). A meta-analysis on Asian populations reported an association between the DAZL A386G mutation and the risk of male infertility (Nejati and Karimian 2016). In brief, mutations A260G, T54A, A386G, and A237G in the DAZL gene are worth investigating further in male infertility.

3.2.3.7 FAS/FASL Gene

Expressing the Fas receptor on their surface, Sertoli cells help in maintaining the population of germ cells by a mechanism that triggers killing of the Fas-positive germ cells, and in case of damage or injury to the Sertoli cells, increased Fas expression carries out self-elimination of nonfunctional Sertoli cells with a smaller number of germ cells (Zhang et al. 2018). FAS-670A/G and FASL-844C/T are the most frequently studied polymorphisms in male infertility, finding that FASL-844 TT and CT genotypes are at a higher risk of idiopathic azoospermia as compared to those having a CC genotype (Asgari et al. 2017). The distribution of both polymorphisms (FASL-844C/T and FAS-670A/G) in dominant, recessive, and allelic models was not significantly different between cases and controls (Asgari et al. 2019). To conclude the possible association between FAS/FASL polymorphism and male infertility, meta-analyses have been done finding nonsignificant association of FAS/FASL variants with infertility (Tüttelmann et al. 2007). Therefore, through these vigorous analyses, it can be concluded that FAS/FASL polymorphisms might not be important for compromised fertility in Asians as well as in other ethnic groups, as these do not show any significant association with male infertility.

3.2.3.8 DNA Polymerase Subunit Gamma (POLG) Gene

The POLG gene in humans encodes for DNA polymerase gamma, which plays an important role in the biogenesis and replication of mitochondria (Wong et al. 2008).

The analysis of the triplet repeat (CAG) polymorphism in this gene revealed a significant association of the 10-CAG allele with infertility in males (Westerveld et al. 2008). Infertile individuals had significantly higher rates of heterozygous and homozygous mutant genotypes (not10/10 and not10/not10) as compared to fertile controls (Baklouti-Gargouri et al. 2012). Although other studies conducted on CAG repeat variants of the POLG gene in different ethnic groups, i.e., South Indian, failed to establish a significant association with male infertility (Poongothai 2013). A meta-analysis also indicated no significant association between POLG-CAG-repeat variants and male infertility in Chinese Han populations (Liu et al. 2011). Therefore, to draw any conclusion about infertility risk linked with polymorphisms in POLG, additional research is required. But at present, analysis of CAG repeat polymorphism in the POLG gene in infertility is not recommended.

3.2.3.9 Estrogen Receptor (ESR) and Male Infertility

In addition to androgens and androgen receptor, estrogens and estrogen receptor also play important roles in spermatogenesis. Several studies have analyzed the connection between estrogen receptor gene polymorphisms and male infertility by investigating the distribution of ESRα (PvuII, XbaI) and ESRβ (RsaI, AluI) polymorphisms in infertile and fertile men (Aschim et al. 2005; Safarinejad et al. 2010). Men having genotypes PvuII TT, XbaI AA, RsaI AG, and AluI AG have a higher probability of having decreased sperm count, lower density, impaired motility, and a lesser percentage of sperm with normal morphology; hence, they are at a higher risk of infertility (Safarinejad et al. 2010). Men with a history of cryptorchidism have three times the frequency of the RasI AG genotype as compared to fertile controls, but this difference holds no statistical significance. Andrological examination of infertile Italian men established a link between the $(TA)_n$ repeat polymorphism in the promoter region of the ESRα gene, finding that men with genotype A (higher TA repeats) had lower sperm counts (Guarducci et al. 2006). High-throughput sequencing aided in discovering eight novel mutations in the ESRβ gene in infertile Indian males, two (g.125C > T and g.126G > C) in the 5' UTR, one (g.11574G > C) in exon 1, two (g.33666G > A and g.33708G > A) in exon 4, two (g.59281C > T and g.59346G > A) in exon 7, and one (g.59432G > A) in intron 7 (Khattri et al. 2009), but none of these showed a significant association with male infertility in Indian men (Khattri et al. 2009). Overall, male infertility risk was substantially correlated with ESR1 PvuII and ESR2 RsaI polymorphisms. The ethnic subgroup analyses showed that both ESR1 PvuII and ESR2 RsaI polymorphisms enhanced the susceptibility to male infertility in Caucasians. Also, ESR1 PvuII and ESR2 RsaI polymorphisms were substantially related to increased infertility risk in Asians. In terms of the ESR2 AluI polymorphism, neither the overall study nor the subgroup analyses using ethnicity/genotyping techniques found any conclusive associations (Ge et al. 2014). Therefore, we can conclude that only a specific allelic combination (RsaI AG and PvuII TT) in the ESR gene may lead to compromised spermatogenesis.

3.3 Diagnostic Techniques

Several strategies can be used to carry out genetic screening. Cytogenetic abnormalities can be identified by karyotyping and fluorescence in situ hybridization (FISH) analysis, which uses chromosome-specific DNA probes. This technique was discovered in 1990 for faster, cheaper, and easier detection of aneuploidies in human sperm. Y-chromosome microdeletions can be identified with the help of PCR. On the other hand, CFTR mutations are detected by Sanger sequencing of amplicons generated by PCR (Bergougnoux et al. 2019). Similarly, single-nucleotide polymorphisms in other autosomal genes are detected with the help of Sanger sequencing.

Recent advancements in technologies have aided in the in-depth study of the etiology of various disorders. In contrast to screening a single mutation in a gene at a time, next-generation sequencing allows the evaluation of the whole genome in a single experiment. Various genome-wide association studies (GWAS) have analyzed a number of polymorphisms simultaneously (Ikegawa 2012). In unique and interesting cases or familial cases of male infertility, exome sequencing and whole genome sequencing can be undertaken using NGS-based methods of DNA sequencing. NGS can also be utilized in analyzing specifically customized gene panels for assessing infertility risk (Fig. 3.2). For example, a gene panel consisting of 87 genes has been developed to diagnose sex chromosome aneuploidies, SNVs/indels, CFTR IVS8-5T variants, copy number variants (CNVs), and YCMD with >90% sensitivity and specificity (Patel et al. 2018). Lorenz et al. (2020) successfully designed a gene panel to assess 75 genes associated with male and female infertility (Lorenz et al. 2020). Another panel consisting of 65 prediagnostic genes has been developed by Precone and colleagues to detect the genetic causes of male infertility (Precone et al. 2020). AURKC, CATSPER1, SUN5, DNAH1, and SYCP3 are some of the notable genes included in this panel (Cannarella et al. 2020; Precone et al. 2020).

3.4 Conclusion and Future Perspectives

In conclusion, genetic testing for SNPs and chromosomal abnormalities has revolutionized our understanding of male infertility. Some of the notable mutations in the CFTR (IVT8-5T), AR (CAG repeats), ESR (RsaI AG and PvuII TT), GSTT1 and GSTM1 (null mutations), and MTHFR (677C/T) genes show significant association with male infertility across all populations (Fig. 3.2), while some other polymorphisms such as GSTP1 (313 A/G) and MTHFR gene (1298A/C) show ethnic-specific association with infertility. Genetic testing panels designed on the basis of polymorphisms significantly affecting infertility risk can be employed to assess the risk of infertility in general population and understand the etiology of infertility in patient population. Genetic testing in patients undergoing treatment would help in better planning of therapy and offering genetic counseling to the affected families. Further research in this field would identify more genes and



Fig. 3.2 Notable mutations in the genes showing significant association with male infertility in various ethnic groups

polymorphisms which affect the risk of infertility significantly. As the number of studies from different ethnicities builds up, ethnic specific analysis can be undertaken to design population-specific genetic testing panels, which can pave way to personalized therapy. Genome-wide studies based on next-generation sequencing would facilitate screening of more polymorphisms for their potential association with infertility, which would accelerate the development of genetic testing panels for infertility in the future.

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Sperm DNA Fragmentation Testing in Infertility

4

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Abstract

Sperm DNA fragmentation (SDF) refers to breaks or damage in the DNA strand (s) in sperm cells, which can lead to an increased risk of male infertility. The extent of DNA fragmentation can vary widely, from mild to severe, and can be caused by a number of factors, including oxidative stress (OS), environmental toxins, and genetic abnormalities. SDF testing can help identify potential fertility issues even in men who have normal semen parameters or unexplained infertility. Moreover, SDF testing can also help predict the success of certain fertility treatments. For example, men with high levels of DNA fragmentation may be less likely to have success with in vitro fertilization (IVF) but may benefit from intracytoplasmic sperm injection (ICSI). SDF analysis is a relatively new field of study, and there are several different methods used to measure sperm DNA fragmentation. Each test has its advantages and limitations, and the results may vary depending on the specific circumstances of the individual being tested. At a time of constantly emerging ideas, policies, and protocols for SDF testing in male infertility, it is important to have an updated understanding of the contemporary SDF tests to ensure that research, diagnoses, and treatment are based on the latest concepts in this field. The present chapter describes updated concepts on SDF and the significance of SDF testing to better identify the underlying mechanisms of

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male infertility, develop tailored male infertility treatment, predict treatment success, and reduce the risk of unsuccessful pregnancy and ART outcomes.

Keywords

Comet assay · Male infertility · SDF tests · Sperm chromatin integrity · Sperm DNA fragmentation · TUNEL assay · Genetic testing · Reproductive medicine

4.1 Introduction

The primary method for evaluating and identifying male reproductive issues is semen analysis (Jarow et al. 2011). Nevertheless, fertility status cannot be accurately predicted through only the impaired semen parameter (Patel et al. 2018), and thus, these laboratory tests alone are insufficient to determine the fertility status of men. Previous research has demonstrated that male infertility is associated with more than just impaired conventional semen parameters but is also linked to sperm DNA damage, among other parameters (Leuchtenberger et al. 1953). The intricate arrangement of genetic material in human sperm makes them particularly vulnerable to harm, resulting in compromised chromatin integrity and significant fertility issues. Reactive oxygen species (ROS) are major factors in causing damage to sperm DNA. ROS have been extensively studied and found to cause lipid peroxidation of the sperm plasma membrane, leading to sperm DNA fragmentation (SDF). Research has demonstrated that elevated levels of SDF can have negative effects on semen quality and reproductive potential, which cannot be detected through routine semen analysis (Dutta et al. 2020). There are a number of methods for assessing SDF, with the most frequently used being the TUNEL assay (terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling). The next most common methods are the SCD (sperm chromatin dispersion) assay and the SCSA (sperm chromatin structure assay) (Selvam et al. 2020). Global fertility specialists recommend SDF testing for male infertility evaluation, particularly in cases of unexplained infertility, varicocele, or exposure to potential infertility factors (Agarwal et al. 2019). Despite the proven efficacy of SDF, adoption by major reproductive societies remains inconsistent, necessitating further studies on chromatin packaging defects and the clinical utility of SDF testing in the evaluation of male infertility. Thus, this chapter brings forth the rationale for the assessment of sperm chromatin integrity in infertile patients while critically discussing and comparing the applied principles of available methods for the assessment of sperm DNA integrity.

4.2 Significance of Sperm DNA Integrity in Male Fertility

Spermatogenesis involves DNA compaction and modification, including histone-to-protamine replacement, controlling transcription, and translation. Disruptions in DNA repair and chromatin rearrangements, due to torsional stress, cause DNA

damage (Henkel and Franken 2011). ROS, particularly from immature spermatozoa, instigate SDF, increasing germ cell apoptosis. Deficient chromatin packaging heightens ROS-induced disturbances. ROS-activated proapoptotic factors induce SDF, affecting fertility (Sakkas and Alvarez 2010).

4.3 Contemporary Sperm Chromatin Integrity Tests

There are various techniques that can be used to assess the integrity of sperm chromatin and DNA. These include staining methods such as aniline blue and acridine orange, as well as assays such as SCSA, 8-hydroxy-2-deoxyguanosine (8-OHdG), comet, sperm chromatin dispersion (SCD), and TUNEL (Table 4.1). The comparison of the features of these tests is presented below.

4.3.1 Sperm Chromatin Maturation (CM) and Condensation Assessments

Sperm chromatin maturation refers to the process by which sperm DNA becomes more stable and resistant to damage as it matures. This process occurs during the final stages of sperm development in the testes and continues during the journey of sperm through the epididymis (Evenson and Wixon 2006). Sperm chromatin condensation, on the other hand, refers to the degree to which the sperm DNA is packaged or tightly coiled. Proper chromatin condensation is important for maintaining the integrity of sperm DNA and preventing DNA damage. Abnormalities in sperm chromatin maturation and condensation can be associated with male infertility and can also impact the success of assisted reproductive technologies (Bibi et al. 2022). Assessments of sperm chromatin maturation and condensation can be performed using various laboratory techniques.

4.3.1.1 Staining with Toluidine Blue (TB)

Toluidine blue is a metachromatic dye of the thiazine family that has a basic nature and selectively binds to acidic components in tissue. Specifically, this dye has a strong tendency to bind to the phosphate residue in sperm DNA with immature nuclei (Agarwal et al. 2017). To perform this method, air-dried sperm smears are fixed onto a slide using a 1:1 mixture of ethanol and acetone at a temperature of 4 °C for 30 min. Next, the sample is treated with 0.1 N hydrochloric acid at a temperature of 4 °C for 5 min to undergo hydrolysis. The slide is then rinsed in distilled water three times. After that, the sperm smear is stained with a solution of 0.05% toluidine blue (in 30% citrate phosphates or McIlvain buffer at a pH of 3.5) for 5 min. To make the slide preparation permanent, the sample is dehydrated using tertiary butanol twice for 3 min, followed each time by a 3-min treatment with xylene twice. Finally, the stained slide is observed using a light microscope. Healthy sperm heads are stained light blue, while sperm heads with damaged DNA are stained violet due to changes in the color of the dye (known as metachromatic changes) (Erenpreiss et al.

Table 4.1 Evaluation of different methods for assessing sperm DNA fragmentation

Test	Principle	Advantage	Disadvantage
Sperm chromatin maturation (CM) and condensation assessments			
Toluidine blue staining	Toluidine blue has a greater attraction to the phosphate residues on sperm DNA, and this can be observed through the utilization of optical microscopy	Rapid, easy, and affordable	Interobserver variability
Chromomycin A3 (CMA3) assay	Indirectly detects DNA lacking protamine by competitively binding to it and is visualized using fluorescent microscopy	It produces dependable outcomes as it is highly associated with other examinations	Interobserver variability
Aniline blue staining	Assess the level of DNA fragmentation in sperm by staining the acidic DNA strands with the basic dye aniline blue. The dye binds preferentially to histones in the chromatin of mature sperm with normal DNA integrity	It is simple, cost-effective, and can be used for both fresh and frozen semen samples, which makes it a versatile method	Interobserver variability, lack of standardization, sensitivity to fixation, and processing techniques
Sperm DNA fragmentation index (DFI) assessments			
TUNEL assay	The measurement of the enzymatic addition of dUTP to DNA fractures is possible with the application of optical and fluorescent microscopy. This procedure can be conducted utilizing optical microscopy, fluorescent microscopy, and flow cytometry	This method is highly sensitive and reliable, with very little variation among different observers. It can also be conducted using a small number of sperm	Standardization is necessary among different laboratories
SCD test (Halosperm assay)	This involves evaluating the scattering of DNA fragments post denaturation, utilizing both optical and flow cytometry techniques	Simple test	Interobserver variability
SCSA	This test evaluates the vulnerability of sperm DNA to denaturation	A dependable calculation of the proportion of sperm	Necessitates costly equipment such as a flow cytometer and

(continued)

Table 4.1 (continued)

Test	Principle	Advantage	Disadvantage
	and is the cytometric adaptation of the AO test. It employs flow cytometry to analyze the results	cells with DNA damage	personnel with advanced technical expertise
Comet assay	The evaluation of DNA fragments from lysed DNA through electrophoresis, employing fluorescence microscopy	This test can be performed with a very small amount of sperm, and it is both accurate and consistent	An experienced observer is necessary due to the variability among different observers
Measurement 8-OHdG	8-OHdG is a biomarker of oxidative DNA damage. Sperm DNA fragmentation can result from oxidative damage, and measurement of 8-OHdG levels in sperm can provide information about the degree of oxidative damage to sperm DNA	8-OHdG is an established marker of oxidative stress. It is a noninvasive and relatively simple test to perform	It does not directly measure DNA fragmentation and may not be a reliable indicator of sperm DNA damage
Acridine orange dye	The fluorescence of this dye undergoes a metachromatic shift when it binds to single-stranded (ss) DNA. This phenomenon can be observed using fluorescent microscopy	Rapid, easy, and affordable	Interlaboratory variations and lack of reproducibility

TUNEL terminal deoxynucleotidyl transferase dUTP nick-end labeling, *SCD* sperm chromatin dispersion test, *SCSA* sperm chromatin structure assay, *8-OHdG* 8-hydroxy-2-deoxyguanosine

2004). The use of toluidine blue staining is useful for evaluating male fertility, with a specificity of 95% and a sensitivity of 42% at a threshold of 45% (Tsarev et al. 2009). This technique is straightforward and employs inexpensive dyes that are commonly used in morphological analysis. Furthermore, the outcomes of this method are consistent with those of advanced tests such as SCSA and TUNEL, which are used to determine sperm DNA fragmentation (SDF) (Shamsi et al. 2011).

4.3.1.2 Chromomycin A3 (CMA3) Assay

Chromomycin A3 (CMA3) represents an assay to determine chromatin condensation by indirectly measuring protamine deficiency in sperm DNA. CMA3, a guanine cytosine-specific fluorochrome, reveals weak chromatin packaging by competing

with protamine to bind with the minor DNA grooves (Hosseinifar et al. 2015). Thus, spermatozoa with low DNA and low protamination display higher CMA3 binding (Erenpreisa et al. 2003).

To prepare an air-dried sample of sperm, it is first treated with a mixture of methanol and glacial acetic acid (in a 3:1 ratio) at a temperature of 4 °C for 20 min. Next, a solution called CMA3 (dissolved in McIvlain's buffer containing 10 mmol/L $MgCl_2$) is added to the sample for another 20 min. The slide is then rinsed with buffer and mounted using a solution made of equal parts PBS and glycerol. The slide is kept overnight at 4 °C, and then 200 spermatozoa are counted under a fluorescence microscope. Spermatozoa that appear bright yellow or bright green after staining with CMA3 are considered CMA3 positive, indicating that they have inadequate DNA protamination. Conversely, spermatozoa that appear faint yellow or dull green are considered CMA3 negative, indicating that they have high protamination of sperm DNA (Kazerooni et al. 2009). This method demonstrated a high level of accuracy, with a sensitivity of 73% and a specificity of 75%. It is a strong predictor of in vitro fertilization (IVF) outcomes (Esterhuizen et al. 2000). There is a negative correlation between the results obtained from the CMA3 test and both sperm morphology, as well as sperm motility and concentration. Research has revealed that individuals with globozoospermia have noticeably greater levels of CMA3 staining when compared to fertile men (Hosseinifar et al. 2015). The sensitivity and specificity of this technique have a strong association with the outcomes acquired through aniline blue staining (Manicardi et al. 1995). The primary constraint of this test is the variability across different individuals.

4.3.1.3 Aniline Blue Staining

Aniline blue staining is a well-established technique used to evaluate the chromatin structure of spermatozoa. Aniline blue is an acidic dye with a high affinity to bind decondensed proteins from residual histones. The staining method allows for the assessment of DNA integrity and condensation in sperm cells, making it a valuable tool in male infertility diagnosis and treatment (Irez and Durmus 2022). Aniline blue is a dye that selectively stains lysine-rich histones, which are found in mature and compact sperm chromatin. When sperm chromatin is properly condensed, it binds to aniline blue and appears stained. In contrast, uncondensed or immature chromatin, which can result from DNA damage, fails to bind with aniline blue. Consequently, a higher percentage of stained cells in a sample indicate a higher proportion of mature and properly condensed sperm, while a lower percentage of stained cells suggests the presence of abnormal or immature sperm with potential DNA damage. Thus, this staining can distinguish between mature and immature sperm cells in an ejaculate. Overall, aniline blue staining provides a simple, inexpensive, and effective means of assessing sperm quality and DNA damage. However, this assay neither measures SDF nor DNA susceptibility to damage. This test is used only to measure chromatin condensation (Hammadeh et al. 2001).

The semen sample is air dried on a slide and fixed for 30 min in 3% glutaraldehyde diluted in PBS. Then, it is stained in a 5% aqueous acidic (pH 3.5) aniline blue solution and kept for 5 min. The stained slide is observed under bright-field

microscopy, and a total of 200 spermatozoa are counted. If the percentage of aniline blue stained sperm exceeds 25%, then the sample is predicted to have a higher number of sperm with decondensed nuclei (Baker and Liu 1996).

Aniline blue staining is a cost-effective protocol for evaluating sperm nuclear instability and decondensation, but the staining is not homogenous. Despite debates over correlations with individual sperm characteristics, it shows potential as an IVF success predictor and an additional tool in initial male infertility investigations, though its relevance to morphological aberrations in asthenozoospermic samples remains uncertain (Hammadeh et al. 2001).

4.3.2 Sperm DNA Fragmentation Index (DFI) Assessments

Sperm DNA fragmentation index (DFI) is a measure of the amount of damage to the DNA in a man's sperm. The higher the DFI, the more fragmented or broken the sperm DNA is, which can lead to infertility (Liu et al. 2023). Measuring the sperm DNA fragmentation index can be done through specialized laboratory tests, including the sperm chromatin structure assay (SCSA) and the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Esteves et al. 2021). Understanding the sperm DNA fragmentation index can be helpful in identifying potential causes of infertility and developing appropriate treatment plans.

4.3.2.1 Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay

The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay is a type of test used to evaluate the integrity of sperm DNA. This assay is based on the principle that when DNA in sperm cells is damaged, the enzyme terminal deoxynucleotidyl transferase (TdT) can add labeled dUTP to the 3′ ends of the broken DNA strands (Caliskan et al. 2022). In a TUNEL assay for sperm DNA, sperm cells are fixed onto slides and permeabilized to allow for the entry of labeled dUTP. The TdT enzyme is then added along with the labeled dUTP, which incorporates into any broken DNA strands present in the sperm cells. The labeled DNA fragments can then be visualized under a fluorescent microscope, with the intensity of the fluorescence corresponding to the level of DNA damage in the sperm cells (Sharma et al. 2021). This assay can provide information on the quality of sperm DNA, which can impact male fertility. High levels of DNA damage in sperm cells have been linked to reduced sperm motility, impaired fertilization, and an increased risk of miscarriage.

The one-step staining method involves using FITC-dUTP to label DNA breaks prior to flow cytometric analysis. In the TUNEL assay, a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) is used to add deoxyribonucleotides to the 3′ hydroxyl end of DNA. This TdT is labeled with a substrate called deoxyuridine triphosphate (dUTP) (Gupta et al. 2017). So, if the DNA has a higher number of breaks, more TdT molecules with dUTP will attach to it because of the increased availability of free 3' OH ends. This assessment of DNA

fragmentation can be performed using either traditional methods or flow cytometry that can be carried out on a laboratory bench (Sharma et al. 2016).

Discussion of the detailed protocol of the TUNEL assay is important to widen the global application of this method in the assessment of sperm DNA fragmentation. TUNEL uses an APO-DIRECT Kit (BD Pharmingen, CA) that comprises the reaction buffer, TdT, fluorescence fluorescein isothiocyanate (FITC)-dUTP, and propidium iodide/RNase (PI/RNase) stain. Both negative and positive controls are also included in the assay kit, which are non-sperm cells. 3.7% paraformaldehyde is used to fix approximately 2.5 × 106 sperm with a fixation time of about 30 min at 4 °C. The next step is centrifugation of the sample at 300 × *g* for 7 min, which eventually removes the paraformaldehyde. From the centrifuged sample, the supernatants should be discarded, followed by resuspension of the pellets with 1 mL ice-cold ethanol (70% vol./vol.). The sample tubes are kept at a temperature of −20 °C for at least 30 min for storage. To create negative sperm controls, the enzyme terminal transferase is taken out of the reaction mixture. On the other hand, positive sperm controls are made by subjecting the samples to a pretreatment with 2% (v/v) hydrogen peroxide and then incubating them at 50 °C for 1 h. The stain (50 μL) is then added to the sample, followed by an hour of incubation. PI/RNase stain is applied and incubated for 30 min after two washes with 1 mL of rinse buffer. Flow cytometry employs a solid blue laser emitting light at two different wavelengths of 488 nm with a power of 20 mW and 640 nm with a power of 14.7 mW from a red laser diode. Green fluorescence between 480 and 530 nm is detected in the FL-1 channel, and red fluorescence at 640 nm is detected in the FL-2 channel to measure the percentage of TUNEL-positive cells using flow cytometer software with a 1023 channel scale. This method was applied using the C6 Plus flow cytometer and BD Accuri software to generate dot plots (Gupta et al. 2017). The results obtained from the TUNEL assay using the standard flow cytometer (C6) were compared with those obtained using the newer version of the flow cytometer (C6 Plus) after calibrations based on cutoff values, sensitivity, and specificity. The performance of both cytometers was observed using two identical sperm preparations and matched settings (Sharma et al. 2019). After adjustment of the settings, there was strong overall concordance, with both cytometers showing 100% positive and 100% area under the curve for negative predictive value. The correlation between the standard (C6) and the newer version (C6 Plus) of the flow cytometer was highly significant ($P < 0.0001$; $r = 0.992$; 95% CI 0.982–0.997). Furthermore, after adjustment, both methods demonstrated high precision and accuracy of 98% and more than 99%, respectively. Strong interobserver agreement on the results from the two instruments established their robustness in assessing sperm DNA fragmentation (Sharma et al. 2019).

Mitchell et al. enhanced TUNEL assay sensitivity for DNA fragmentation detection in spermatozoa by incubating cells in 2 mm DTT for 45 min and then fixing with formaldehyde. They also introduced LIVE/DEAD far-red stain incubation, permitting concurrent evaluation of DNA integrity and cell vitality (Mitchell et al. 2011).

Male infertility research widely uses the TUNEL assay for measuring sperm DNA fragmentation (Carrell et al. 2003). SDF proves effective as an ART outcome

predictor, influencing ICSI fertilization, IUI pregnancy, and IVF embryo cleavage rates. The SDF test offers a scientific comprehension of the root cause of repeated miscarriages (Benchaib et al. 2003). A predictive limit of 19.2% has been determined that can effectively differentiate between men who are fertile and those who are not. This distinction can be made with a sensitivity of 64.9% and a specificity of 100% (Benchaib et al. 2003). This value supersedes the prediction threshold for IUI procedures, which is only 12% (Sun et al. 1997). The TUNEL assay, with a 16.8% cutoff, exhibited high specificity (91.6%) and positive predictive value (90%) for detecting male infertility sans sperm DNA fragmentation. The high specificity of the TUNEL assay is helpful in ensuring accurate diagnosis (Sharma et al. 2016), while the strong positive predictive value of the TUNEL test can be used to confirm that the presence of SDF is the reason for infertility in men who have tested positive (Cui et al. 2015).

The TUNEL assay, costly and laborious, displays variability in results due to fixative properties, incubation time, DNA fluorochrome, and data analysis methods. Flow cytometry outperforms fluorescent microscopy in accuracy yet incurs greater expense and complexity, with SDF reproducibility issues (Muratori et al. 2010). Benchtop flow cytometry TUNEL testing showed high consistency, yielding below 8% observer variation (Sharma et al. 2010).

4.3.2.2 Sperm Chromatin Dispersion (SCD) Test/Halosperm Assay

The sperm chromatin dispersion (SCD) test (Halosperm assay) is a diagnostic test that evaluates the quality of sperm DNA by measuring the ability of sperm chromatin to disperse in a halo-like pattern (Esteves et al. 2022). SCD shows that sperm nucleoids that contain core and peripheral halo result from DNA loop release, and this denotes that the sperm is devoid of DNA fragmentation. Upon treatment of sperm samples with an acid solution followed by lysis buffer, minimal or absolutely no halo will be seen if the spermatozoal DNA is fragmented. On the contrary, the test produces a distinct halo when spermatozoa possess DNA with high integrity (Fernández et al. 2003). When sperm with non-fragmented DNA is submerged in an agarose matrix and exposed directly to lysing solutions, deproteinized nuclei (nucleoids) are produced, which exhibit extended halos as observed under bright-field or fluorescent microscopy. If DNA breaks are present in sperm, the nucleoid halo will be extended (Muriel et al. 2006).

In the SCD test, sperm aliquots are prepared with phosphate buffer solution (PBS) at a concentration of 5–ten million/mL. Specimen are then mixed with aqueous agarose (1% low melting point at 37 °C), aiming for a 0.7% final concentration of agarose. 50 μL aliquots of the prepared sample are added to a glass slide (precoated using 0.65% standard dried agarose at 80 °C), covered with a glass coverslip, and kept undisturbed for 4 min for it to solidify at 4 °C. The process involves immersing the slides in a newly prepared acid solution containing 0.08 N HCl for denaturation, which is done in the dark for 7 min at 22 °C. This causes the DNA to break, leading to the formation of single-stranded DNA (ssDNA) motifs. Next, the slides are placed in a tray with solution 1, which contains Tris, DTT, SDS, and EDTA, for 10 min to neutralize and lyse the proteins. The slides are then incubated in solution

2, containing Tris, NaCl, and SDS, for 5 min. Afterward, the slides are washed in Tris-borate EDTA buffer, dehydrated in increasing concentrations of ethanol, and air-dried. In the newer version of the SCD test (Halosperm® kit), the slides are horizontally smeared with Wright's staining solution for bright-field microscopy. If the slide is to be observed under fluorescence microscopy, the cells are stained with 2 μg/ml of DAPI (4′,6-diamidino-2-phenylindole) (Zini et al. 2002).

The advantages of the SCD test include its speed, straightforward protocol, and ability to produce reproducible results that are comparable to those obtained from the SCSA and TUNEL tests. Additionally, the SCD test is cost-effective, because it can be performed using bright-field microscopy, which reduces equipment expenses (Muriel et al. 2006). The SCD test is also cost-effective as the protocol supports the use of bright-field microscopy, which significantly decreases equipment costs. This test has been shown to be convenient for use in clinical studies for the determination of sperm DNA damage (Meseguer et al. 2009) as well as for its potential merger with the fluorescent in situ hybridization (FISH) assay, termed together as the SCD-FISH assay to detect sperm aneuploidy (Balasuriya et al. 2011). This represents the sole test that allows simultaneous determination of SDF and chromosomal aneuploidy in the same sperm via FISH. When the SCD test is combined with the additional step of incubating sperm with an 8-oxoguanine DNA probe, oxidative damage to DNA can also be determined. This procedure can easily be performed using the Halosperm kit (Fernández et al. 2011). Research studies indicate that there is a negative correlation between SDF (sperm DNA fragmentation) as measured through SCD (sperm chromatin dispersion) testing and ART (assisted reproductive technology) outcomes, specifically in terms of fertilization rates and embryo quality. However, the studies did not find any link between SDF and the rates of clinical pregnancy or live births (Muriel et al. 2006).

4.3.2.3 Sperm Chromatin Structure Assay (SCSA)

The sperm chromatin structure assay (SCSA) measures the susceptibility of sperm DNA to acid-induced denaturation, which reflects the degree of DNA fragmentation. Sperm samples are mixed with an acid solution and then analyzed using a flow cytometer, which detects changes in the sperm chromatin structure (Evenson 2022).

SCSA uses acridine orange (AO) fluorescence staining to measure the level to which in situ DNA is susceptible to conformational changes in the helix coiling caused by acid or heat. This level of sperm DNA conformational alterations is detected through the measurement of the AO fluorescence metachromatic shift from green, as in the case of native supercoiled DNA, to red, which indicates relaxed-coiled or denatured DNA. Acid-induced SCSA protocols are more convenient to use than heat treatments. SCSA-defined sperm DNA damage is referred to as the DNA fragmentation index (DFI) (Evenson et al. 1999).

To prepare for flow cytometric analysis, a small portion of the semen sample, approximately 13–70 μL, is mixed with TNE buffer consisting of 0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM EDTA at pH 7.4. The mixture is then diluted to a concentration of 1–2 × 106 sperm/mL. After that, an acid detergent solution containing 0.1% Triton X-100, 0.08 N HCl, and 0.15 mol/L NaCl at pH 1.2 is

added to the sperm suspension and allowed to react for 30 seconds. The sample is then stained with 6 mg/L purified AO in a phosphate-citrate buffer with pH 6.0. Finally, the stained sample is analyzed using flow cytometry (Evenson et al. 1999), and the detected level of DNA fragmentation is measured as the percentage of sperm fragmented DNA in the sample, which is termed as the DNA fragmentation index (DFI). The flow cytometric detection is inferred as such that the DFI represents the ratio of red fluorescence (indicating fragmented DNA) to the sum of red and green (native or intact DNA) fluorescence. SCSA is a preferred test for epidemiological studies of male infertility owing to its consistent results over a long duration as compared to routine semen analysis following the World Health Organization (WHO) guidelines (Spanò et al. 1998). While DFI shows potential as an index to detect sperm DNA damage in male infertility and its clinical threshold has been determined, there is no evidence to support an age-related rise in DFI. SCSA can also be used to categorize men as per their fertility status, as such high fertility corresponds to impregnation of the partner within 3 months, moderate fertility corresponds to impregnation within 4–12 months, and unproven fertility refers to no pregnancy attainment after a duration of 12 months. It has been suggested that if a subject's semen samples have a DFI threshold of under 30%, it may indicate that their ability to initiate a pregnancy in vivo is also under 30% (Bungum et al. 2007).

SCSA can be used for the prediction of ART outcomes (Mona Bungum et al. 2008), such as fertilization and implantation rates (Bungum et al. 2007). Nevertheless, a study by Lin et al. (2008) contradicted these reports and showed that DFI of even more than 27% corresponded to an increased abortion rate and that SCSA results could not be significantly correlated with IVF and ICSI outcomes (Collins et al. 2008). Thus, SCSA has been proposed to be used as a supplement test besides the standard routine semen analysis. The technique requires costly equipment (a flow cytometer) and a high level of technical proficiency. It has been suggested that the TUNEL assay is superior to the SCSA method, because the former enables a more precise measurement of sperm DNA damage, whereas the latter estimates the susceptibility to DNA damage (Henkel et al. 2010).

4.3.2.4 Comet Assay

The technique known as the comet assay, or single-cell gel electrophoresis, uses the basic principles of DNA permeability and electrophoretic movement. The comet assay evaluates the integrity of sperm DNA by measuring the amount of DNA damage, represented by the comet-shaped appearance of the DNA when it is subjected to an electric field (Serrano Berenguer et al. 2022). In the neutral version of the assay, loops of double-stranded DNA move out from a relaxed, supercoiled nucleus of a damaged cell, forming a "tail" of unwound DNA whose length reflects the degree of cellular damage. This tail has a unique "comet" appearance when viewed under a fluorescence microscope after staining. Singh et al. (1988) later modified the assay by using alkaline electrophoresis buffers to expose alkali-labile DNA sites (Singh et al. 1988). The modified two-tailed comet assay was developed to improve test sensitivity in detecting both single-stranded (ss) and double-stranded (ds) DNA breaks. The majority of SDF tests cannot identify and distinguish between

ssDNA breaks (SSB) and dsDNA breaks (DSB) in the same sperm cell, but the two-tailed comet assay (2T-Comet) procedure can. This test evaluates DNA damage by measuring the distance between the nucleus (comet head) and the extended DNA tail, with tail length being an indicator of sperm damage. The "tail moment" or twisting force of the tail is calculated by multiplying the tail length with the percentage of total DNA in the tails (Hellman et al. 1995).

The benefits of comet assay are that it is convenient, sensitive, fast, and versatile assay (Benchaib et al. 2003). Although the two-tail comet assay is a reliable method for measuring and describing sperm DNA damage, it requires a significant level of expertise to interpret the results by analysing observations obtained through fluorescence microscopy (Enciso et al. 2009).

The comet assay has been demonstrated to be a useful method for assessing the extent of DNA damage in sperm that has undergone cryopreservation (Duty et al. 2002). The outcomes of this examination have been utilized to forecast the growth of embryos subsequent to assisted reproductive technologies, particularly in situations where the cause of infertility is not known. Although clinical thresholds for the comet assay have been established for diagnostic purposes related to infertility and assisted reproductive technology outcomes, there is still no complete agreement regarding its connection with clinical pregnancy outcomes (Simon et al. 2011).

4.3.2.5 Measurement of 8-Hydroxy-2-Deoxyguanosine (8-OHdG)

This test measures 8-hydroxy-2-deoxyguanosine (8-OHdG), a substance that is produced when sperm DNA undergoes oxidative damage. It is used to determine the extent of oxidative damage to cells. 8-OHdG is the most commonly researched indicator of oxidative damage to DNA due to its strong mutagenic effects, specificity, and abundance within DNA (Shen and Ong 2000).

This method involves a three-step procedure. In the first step, cells are washed with a buffer solution containing Tris-HCl, EDTA, NaCl, and a pH of 7.0. Then, the cells are lysed with a mixture of SDS, proteinase K, and DTT at 55 °C for an hour, followed by the use of chloroform-isoamyl alcohol to extract sperm DNA. RNA is removed using ribonuclease A, and the extracted sperm DNA is digested in Tris-HCl at a pH of 7.0. The second step involves enzymatic DNA digestion, which is carried out with the help of three enzymes: DNase I, nuclease P1, and alkaline phosphatase. The resulting solution is then dried at low temperature and pressure and redissolved in deionized distilled water. The third step involves high-performance liquid chromatography (HPLC) analysis, which requires equipment such as a pump, a Partisphere 5C18 column, an electrochemical detector, a UV detector, an autosampler, and an integrator. The mobile phase for the HPLC analysis is made up of NH4H2PO4, EDTA, and methanol and has a pH of 4.7. Standard 8-OHdG is used to calibrate the curves for 8-OHdG, and the unit of measurement is 8-OHdG/104 dG (Kodama et al. 1997). The OxiDNA assay kit includes a fluorescent probe called 8-oxoG, which can be utilized to identify 8-oxoG as well.

The 8-OHdG assay is among the direct tests for the detection of oxidative damage to sperm DNA in infertile men and can also distinguish distinctly between fertile and infertile men based on the levels of 8-OHdG in spermatozoa (Kodama et al. 1997).

Studies have demonstrated a positive association between the measurement of SDF using the TUNEL assay and the production of 8-OHdG. 8-OHdG levels also positively correlate with impaired sperm chromatin remodeling (De Iuliis et al. 2009). The concentration of a crucial marker for oxidative damage to sperm DNA has been found to be elevated in male smokers but can be reduced by increased intake of vitamin C and higher levels of vitamin C in semen. Infertile patients with varicocele have been shown to have higher levels of 8-OHdG and impaired sperm production (Ishikawa et al. 2007). 8-OHdG alterations in DNA have been reported to be mutagenic, which should be repaired or they may lead to undesired consequences, such as miscarriages, fetal malformations, or even childhood malignancy (Agarwal et al. 2009).

4.3.2.6 Acridine Orange Dye

Acridine orange, a fluorescent dye, is used to evaluate sperm nuclear DNA vulnerability to acid-induced denaturation. It exhibits green fluorescence with native DNA but emits red light when bound to denatured DNA (Mohammed et al. 2015). Fluorescence microscopy or flow cytometry enables sperm detection. Carnoy's fixative treats thick semen smears before acridine orange staining. Post-rinsing with deionized water, analysis of ~200 spermatozoa discerns native (green fluorescence) and damaged DNA (yellow-orange to red fluorescence) (Kazerooni et al. 2009). DFI is obtained by the ratio of (yellow to red)/(green + yellow to red) florescence (Mohammed et al. 2015). To use flow cytometry for detection, a sample containing one million sperm in a volume of 25 to 100 μL is mixed with ice-cold PBS (pH 7.4) and centrifuged at 600 × *g* for 5 min. The resulting pellet is then resuspended in an ice-cold TNE solution (consisting of 0.01 mmol/L of tris-HCl, 0.15 mol/L of NaCl, and 1 mmol/L of EDTA at pH 7.4) and centrifuged again for 5 min at 600 × *g*. After discarding the supernatant, the pellet is resuspended in a TNE solution containing 10% glycerol and fixed in 70% ethanol for 30 min. Next, the sample is treated with a solution of triton-X 100 (0.15 mol/L of NaCl and 0.08 N HCl at pH 1.2) for 30 s, followed by the addition of acridine orange. The sample is then analyzed using flow cytometry with a light source at 488 nm, and 5000 cells are evaluated. The acridine-bound native dsDNA emits green fluorescence (at 513–530 nm), while the acridine-bound damaged ssDNA emits red fluorescence (at 630 nm) (Gorczyca et al. 1993).

In infertile men, acridine orange-positive sperm cells indicate greater potential for DNA damage and structural abnormalities. The repair of varicocele reduces DNA damage susceptibility, enhancing its clinical utility. With low variability, it's reproducible and distinguishes infertility at a 20–50% cutoff (Evenson et al. 1999). Single-stranded DNA staining inversely relates to successful IVF and pregnancy outcomes (Hoshi et al. 1996).

4.4 Impediments in Sperm Chromatin Integrity Testing

4.4.1 Limiting Factors in Sperm Chromatin Integrity Testing

SDF test standardization has faced criticism due to interlaboratory variability (Esteves et al. 2017a, b). The tests fall into three categories: direct and indirect measures, tests that assess chromatin compactness, DNA breaks before and after denaturation, and those that use DNA probes or modified nucleotides to detect SDF at damage sites, complicating comparison of results (Esteves et al. 2017a, b). Suggested SDF cutoff values exist but are not universally accepted. Some tests showed a correlation between increased SDF levels and infertility, with a 91.4% positive predictive value and a 33.1% negative predictive value (Sharma et al. 2016). Other studies proposed specific DFI cutoff values, differentiating fertility status or IVF/ICSI outcomes (Lopes et al. 1998). However, SDF testing lacks precision in identifying sperm DNA characteristics and location. Sperm chromatin decondensation degree is predicted by aniline blue and toluidine blue tests, whereas TUNEL, SCSA, and SCD more accurately measure DNA fragmentation (Esteves et al. 2017a, b). Unlike ARTs, SDF tests use raw semen; sperm manipulation during processing may adversely affect DNA integrity (Esteves et al. 2017a, b).

4.4.2 Disparity in Universal Predictive Value for Sperm DNA Fragmentation

Limitations of SDF testing make it a less favorable option for patients with unexplained infertility, repeated pregnancy loss, and poor assisted reproductive technology (ART) outcomes (Esteves et al. 2017a, b). Despite its prevalence, sperm DNA damage only showed a 42.9% incidence via SCD test for couples undergoing IUI due to unexplained infertility (Vandekerckhove et al. 2016). Its predictive value for IVF and ICSI outcomes is still disputed, as SDF is influenced by many ART factors (Esteves et al. 2017a, b). High DFI negatively correlates with sperm motility, and research suggests SDF negatively impacts clinical pregnancy rates after IVF and ICSI (Simon et al. 2017). SDF levels might guide fertility experts in deciding varicocelectomy for men with normal semen parameters, and repairs have shown reduced SDF levels (Esteves et al. 2017a, b). Varicocelectomy has also led to improved DFI and semen parameters in several studies (Abdelbaki et al. 2017). Still, SDF test accuracy can be affected by various factors (Agarwal et al. 2017a). Lifestyle modifications and antioxidant use may improve DFI and sperm functionality, but more research is needed to evaluate their effectiveness in treating SDF in infertile men.

4.5 Conclusions

- SDF testing serves as a consequential tool in diagnosing male infertility, especially in scenarios where traditional semen analyses fail to provide an explanation. It gains significance in instances of recurrent ART failures, with numerous cases showing elevated DNA fragmentation despite normal semen parameters.
- Among the range of SDF tests, the SCSA and the TUNEL assay are identified as the most robust, sensitive, and reproducible, with selection influenced by the clinical scenario and laboratory expertise. Despite its utility, SDF testing should not be indiscriminately used due to potential patient anxiety and financial impact; its application is best reserved for specific clinical indications, such as unexplained infertility, recurrent miscarriages, or repeated ART failure (Fig. 4.1).
- High SDF has been correlated with diminished fertility potential, while its reduction is associated with increased success rates in intrauterine insemination (IUI), IVF, and ICSI. Therefore, the inclusion of SDF testing in infertility evaluation, particularly in cases of multiple ART failures, is advocated to enhance fertility treatment strategies, consequently augmenting the potential for successful infertility treatment.

4.6 Future Perspective

The Society for Translational Medicine advocates for professional societies to lead in revising clinical guidelines related to sperm DNA fragmentation (SDF) testing, an essential tool for understanding male infertility (Agarwal et al. 2017b). Optimal selection of patients for SDF testing and its incorporation alongside conventional semen analysis can refine diagnostic accuracy and optimize treatment strategies, thereby improving reproductive outcomes. Notably, SDF testing is highly predictive in unexplained infertility scenarios and boosts the success rate of assisted reproductive tsechnologies (ARTs). Appropriate SDF assay selection hinges on protocol complexity, equipment cost, technician proficiency, and andrology lab capabilities. Understanding sperm DNA damage level and cause is crucial for suitable therapeutic measures, minimizing potential adverse effects on offspring. Varied factors induce strand breaks, leading to fertility issues. Ineffectual DNA repair correlates with impaired sperm and increased genetic anomalies risk. SDF testing in specific cases can aid infertility treatment decision-making (Sakkas et al. 1998).

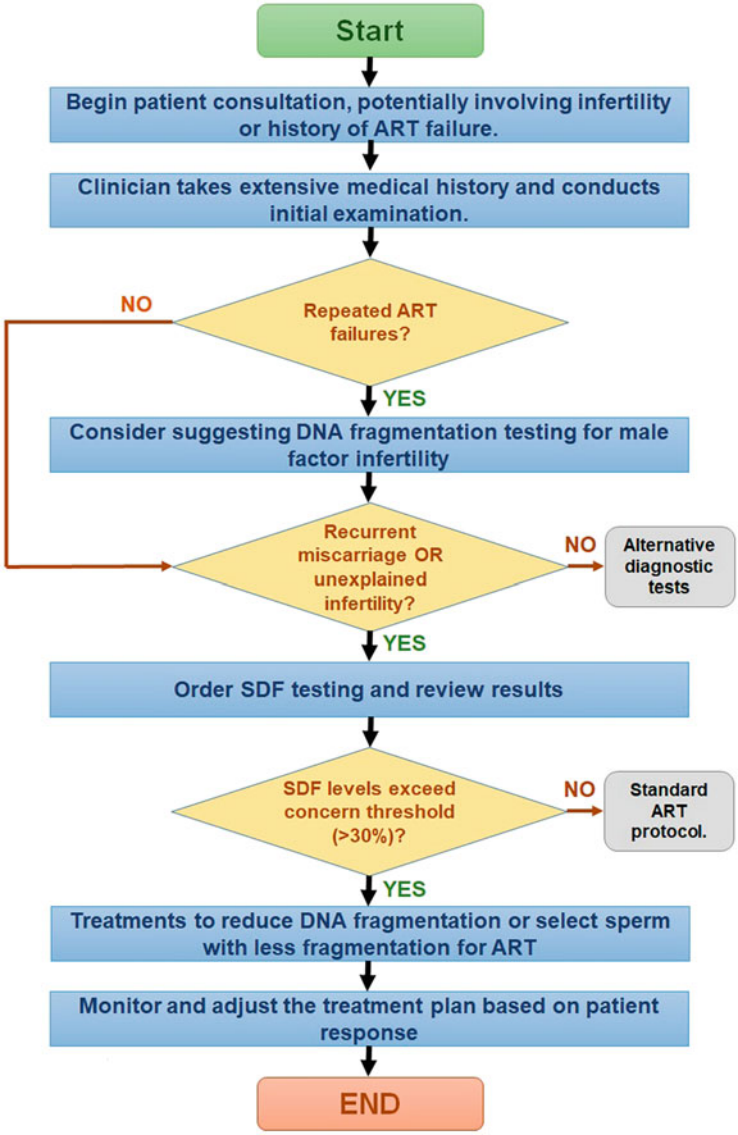

Start
Begin patient consultation, potentially involving infertility or history of ART failure.
Clinician takes extensive medical history and conducts initial examination.
Repeated ART failures?
NO
YES
Consider suggesting DNA fragmentation testing for male factor infertility
Recurrent miscarriage OR unexplained infertility?
NO
Alternative diagnostic tests
YES
Order SDF testing and review results
SDF levels exceed concern threshold (>30%)?
NO
Standard ART protocol.
YES
Treatments to reduce DNA fragmentation or select sperm with less fragmentation for ART
Monitor and adjust the treatment plan based on patient response
END

Fig. 4.1 Flowchart for SDF testing decision-making in male infertility cases. It begins with patient consultation, medical history, and examination (Step 1), leading to Decision Points A and B, assessing repeated ART failures, and the risk factors for DNA fragmentation, respectively. DNA fragmentation testing is done if criteria are met (Steps 2 and 3), followed by analysis (Step 4). Based on the results (Decision Point C), appropriate treatments (Step 5) are selected, the plan is monitored, and adjustments are made (Step 6), culminating the process

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Microbiome Testing in Male Infertility

5

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Abstract

Infertility is a worldwide problem for couples of reproductive age. The etiologies are diverse, but approximately 50% are attributable to the male factor. The explosion in the "-ics" fields, including genomics, epigenetics, proteomics, metabolomics, and recently microbiomics, has shed light at molecular and metabolic levels on unknown causes of various infertility problems. The microbiota is mainly a community of bacteria, viruses, and fungi and in combination and interaction with the surrounding microenvironment form the whole human and animal microbiome. The study of the human microbiome has been of great interest in the last decade, since it has been related to infertility problems in both men and women. The microbiota and its relation to human reproduction are the tip of the iceberg in an unexplored sea. On the other hand, the relationship between the reproductive microbiome and assisted reproduction is of clinical significance, as reflected in the clinical results of IVF treatment. The present chapter is primarily concerned with the male microbiome, the potential impact of dysbiosis on male fertility, and the methods of and need for microbiome analysis in male infertility treatment.

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Semen microbiota · Male infertility · Semen microbiome · Genetic testing · Microbiome analysis · Reproductive medicine

5.1 Introduction

Looking at the WHO 2009 and WHO 2021 reports, it can be observed that there is a steady decrease in the lower limit that determines the quantitative and qualitative characteristics of a normal semen sample. Despite these changes in sperm parameters highlighted by the WHO, there are high-quality studies that have shown an opposite trend (Jørgensen et al. 2012). Nevertheless, the majority of studies reveal that there is a decline in sperm parameters, attributable mainly to lifestyle and environmental factors, including obesity, diet, smoking, alcohol, and toxins (Mann et al. 2020). Apart from the primary causes of male infertility, such as congenital anomalies or endocrine disorders, there is also idiopathic infertility and secondary elevated DNA fragmentation or oxidative stress. However, male infertility remains one of the unresolved issues requiring attention.

In the era of "-ics," including microbiotics, research has focused on the microbiome, since it has been referred to as an important factor that has been overlooked in male infertility (Wang et al. 2022). Although in previous years it was assumed that the presence of bacteria in semen was a clear sign of infection, it was not until the era of next-generation sequencing methods that it was revealed that human semen consists of specific microbiota, maintaining a healthy status of spermatozoa (Monteiro et al. 2018). However, *Escherichia coli* (*E. coli*) appears to be one of the most popular and common pathogens causing urogenital infections (Farsimadan and Motamedifar 2020), implying indirectly a possible negative effect on sperm parameters and male fertility (Berjis et al. 2018). Indeed, the presence of *E. coli* in sperm samples has been associated with reduced sperm quality parameters (Vicari et al. 2016). The mechanism underlying this effect seems to be either the direct adhesion of *E. coli* to the sperm cell or the expression of hemolysin A factor, which is responsible for sperm DNA damage (Lang et al. 2013). Bearing in mind that semen volume consists mainly of secretions of the accessory glands containing lipids, proteins, glycans, and inorganic ions, this environment seems to be ideal for the growth of *E. coli* and other pathogens of the microbiome spectrum (Javurek et al. 2016; Jodar et al. 2016). In line with *E. coli*, another popular microorganism that is closely related to male infertility is *Staphylococcus aureus* (*S. aureus*), which belongs to the *Staphylococcal* species. The latter as well as the *Lactobacillus* species (mentioned below) are among the most common components of the microbiome of the seminal fluid (Chen et al. 2018; Monteiro et al. 2018; Amato et al. 2019). Regarding *S. aureus*, it was found that it is the causing factor for male reproductive dysfunction, such as the reduced percentage of sperm motility along with reduced sperm concentration (Esmailkhani et al. 2018). Moreover, in a study investigating a number of bacteria, including *S. aureus*, it was found that the combined presence of

bacteria in the seminal fluid was associated with lower sperm concentration and an increased percentage of sperm protamine deficiency (Merino et al. 1995). The limitation of that study was the lack of association between each bacterium and male fertility. As far as *Lactobacillus* is concerned, the presence of these species seems to have dual effects on male fertility. Specifically, azoospermic patients demonstrated a high abundance of *Lactobacillus genus* (Chen et al. 2018), while another study in a similar group of patients found a low abundance of *Lactobacilli* (Monteiro et al. 2018). Recently, Yang et al. (2020) showed that asthenozoospermic and oligoasthenozoospermic semen samples are related to the presence of *Lactobacillus*. Due to the fact that *Lactobacillus* species are present in the female reproductive tract, functioning mainly as probiotics and providing protection against other bacteria, it can be assumed that the prevalence of *Lactobacillus* in semen may serve as an agent of the same purpose (Franasiak and Scott 2015a, b), enforcing the notion mentioned before that the presence of bacteria in semen does not necessarily mean a sign of infection. Indeed, *Lactobacilli* appear to prevent sperm lipid peroxidation and consequently preserve sperm motility and viability (Barbonetti et al. 2011).

Although seminal microbiomes may originate from various urogenital tissues, a recent study found that *Staphylococcus* species come from either the testes or the epididymis (Lundy et al. 2021). Nevertheless, a defective gut barrier in combination with the translocation of the microbiome through the blood flow may finally lead to the dissemination of the microbiome into the testis (Wang and Xie 2022). The semen microbiome is not limited to the above-described bacteria. Several studies have raised concerns about *Actinobacteria*, which were prevalent in the biopsy samples (Alfano et al. 2018). Furthermore, the presence of the bacterium *Prevotella* in seminal fluid appears to have a negative effect on sperm parameters, and this observation has been elucidated by several studies (Baud et al. 2019; Lundy et al. 2021).

It was as early as 1967 when *Ureaplasma* species were closely related to male infertility (Kundsin et al. 1967). Since then, both the *Ureaplasma* species, *Ureaplasma urealyticum* (UUR) and *Ureaplasma parvum* (UPA), have been repeatedly isolated from infertile semen specimens, implying that the presence of *Ureaplasma* in semen in high concentrations is one of the main potential factors that may cause male infertility (Huang et al. 2015). This idea was supported by the findings that the presence of UUR was directly associated with reduced sperm parameters, including total sperm count and pH, while the production of reactive oxygen species (ROS) was negatively affected to the extent that the key sperm parameters were compromised (Upadhyaya et al. 1984). Similar to the previous microorganisms, the exact mechanism by which *Ureaplasma* species exert their action on sperm parameters has not yet been defined, and several assumptions are up for discussion. Among them, the direct impact of *Ureaplasma* on the sperm membrane and DNA is the most persuasive, while alterations in the nitric oxide cycle cannot be ruled out (Qian et al. 2016). Although the literature on the association between *Ureaplasma* and male fertility is relatively old, the effect of *Ureaplasma* species on male fertility appears to be a constant concern of the research community, and specifically increased UPA concentrations have been associated with a

decreased percentage of progressive sperm motility (Zhou et al. 2022). In recent studies, it was demonstrated that the prevalence of *Chlamydia trachomatis* and *Mycoplasma hominis* in semen specimens were much higher in infertile individuals when compared to fertile controls (Ahmadi et al. 2017, 2018), supporting the idea that both these microbes are associated with male infertility.

In the definition of the microbiome, the presence of viruses cannot be excluded, although the presence of viruses is linked to infection and not to the symbiosis theory, as is the case with other species. Since viruses are included in the spectrum of microorganisms, their presence either in the seminal fluid or in spermatozoa may compromise male fertility. A number of viruses have been detected in semen, including hepatitis B and C (HBV, HCV), human immunodeficiency virus (HIV), human papillomavirus (HPV), as well as SARS-CoV-2. HBV is an enveloped DNA virus that causes both acute and chronic infections. The global prevalence of HBV infection in the general population is approximately 3.5%. HBV, HCV, and HIV testing are mandatory according to the European Tissues and Cells Directive as a preventative measure to reduce the risks of transmission to their partners and offspring (WHO 2015, 2017, 2018). HBV can be detected inside the sperm cytoplasm, making vertical HBV transmission possible, which has not yet been proven and remains at a theoretical level (Huang et al. 2003). The detection of HBV in the seminal fluid seems to have significance during sperm cryopreservation, but the use of cryoprotectants, culture media, removal of the seminal fluid, and hermetically closed sperm cryovials make the process of cryopreservation completely safe and free of virus particles (Shapiro et al. 2020). The data in regard to the association between HBV detection in semen and male fertility/infertility is scarce, and most of the studies focused on the impact of sperm HBV on IVF outcome, which will be discussed below. Nevertheless, the presence of HBV in semen was associated with reduced sperm motility and viability (Karamolahi et al. 2019; Wang et al. 2021). However, there is a study with contradictory results showing that the presence of HBV in semen does not compromise sperm parameters (Afrakhteh et al. 2021). Currently, there is no study investigating the detection of HCV in sperm; however, the presence of HCV in the seminal fluid can be eliminated by gradient centrifugation followed by multiple washing steps. Good laboratory practices regarding semen processing are recommended for male patients infected with HCV. HCV infection appears to have a negative impact on sperm parameters, including DNA fragmentation and chromatin status (La Vignera et al. 2012; Hofny et al. 2011). Regarding HIV, the presence of the virus on the sperm surface has been demonstrated (Young et al. 2019). Similar to the previous viruses, good laboratory practices are essential for reducing the chance of collecting sperm samples free of the virus. It has been shown that sperm motility is impaired in HIV-positive patients (Frapsauce et al. 2015). HPV, both low-risk and high-risk subtypes of the virus that may cause several threatening diseases, has been detected either on the sperm surface or integrated into the DNA of the sperm host cell. Studies regarding the effect of the virus on sperm parameters are controversial. Nevertheless, the majority of the studies concluded that there is an association between the prevalence of HPV and male infertility (Capra et al. 2022). The COVID-19 pandemic was caused by SARS-CoV-2. The detection

of SARS-CoV-2 in semen samples was a debated issue (Pavone et al. 2020; Holtmann et al. 2020) by many researchers until the majority of the studies by 2022 failed to detect viral loads in semen samples either in the acute phase of infection or in the recovery phase (3 weeks postinfection). Despite the undetectable levels of the virus in semen, the wider implications of infection on male fertility, even if temporary, were a fact.

5.2 Testing of Semen Microbiota

The examination of the composition of human microbiota in several body sites has been suggested as a prerequisite for investigating related health issues (Tett et al. 2021). The evaluation of the semen microbiota for the investigation of male infertility is not routinely considered. It is crucial to clarify that the presence of a microbiome in semen may act in a beneficial manner, as it happens with gut microbiota. When this symbiotic balance is disturbed, for example, due to inflammatory signaling, the symbiotic interaction is turned into dysbiosis, which in turn may affect sperm parameters negatively (Morawiec et al. 2022). The exact balance between symbiosis and dysbiosis has not yet been elucidated, and therefore, the seminal microbiota and male infertility need further investigations. Although several microbes have been associated with normal sperm parameters, such as *Lactobacillus* and *Propionibacterium* species (Okwelogu et al. 2021; Tomaiuolo et al. 2020), there are a number of microbial species, such as *Streptococcus*, *Neisseria*, and *Gardnerella* (Koedooder et al. 2019; Okwelogu et al. 2021) that are closely related to abnormal sperm parameters. For this reason, it is obvious that the testing of the seminal microbiota may play a critical role in the evaluation, diagnosis, and treatment of male infertility.

In the past, the only diagnostic tool for the evaluation of seminal microbiota was culture of the semen sample. However, this testing method was not enough to form a complete opinion about the seminal microbiota, since it was limited to specific bacterial species. Therefore, clinicians were restricted to the positive-negative culture results, without knowing whether there was a symbiosis imbalance. In the era of omics, including transcriptomics, genomics, and metagenomics, the evaluation of seminal microbiota has become far easier and more accurate. Currently, 16S rRNA sequencing is a molecular tool that analyzes the highly conservative sequence of ribosomal 16S for the identification of different species of bacteria. Whole genome and metagenomic sequencing are also widely used as tools for microbial research that accurately characterize the seminal microbiome, including non-culturable species (Koedooder et al. 2019). The use of sequencing methods in seminal fluid revealed that human semen contains various microbial species (Tomaiuolo et al. 2020), whose functions have not yet been clarified. Nevertheless, it is obvious that the impact of microbial dysbiosis and its contribution to male infertility have been underestimated.

5.3 Semen Microbiota, Infertility, and Assisted Reproduction

The relationship between human microbiota and health is undisputable (Tett et al. 2021), implying that there is an increasing global concern about the possible association between microbiota and fertility. Indeed, recently, the gut microbiota has been directly associated with male infertility, meaning that the role of the microbiota has been overlooked (Wang et al. 2022). The "culture-negative" status of the microbiological tests considered free of microbiota did not allow the clinicians to see the problem arising from the presence of microbiota. The symbiosis theory and the possible effects across almost all body sites, including the male reproductive tract, were underestimated to the extent that dysbiosis affects various health parameters, including male fertility. Whether there is an association between the microbiome and IVF outcome, the existing data are limited, and no solid conclusions can be drawn. Nevertheless, a recent study comparing the microbiome of intrauterine insemination (IUI) successful patients to the microbiome of IUI failure patients did not observe significant differences (Amato et al. 2019). Although studies regarding the IVF outcome are lacking, the seminal microbiome, in terms of dysbiosis, appears to impact the quality of embryos negatively (Štšepetova et al. 2020). This finding may not be as innocent as it appears because the semen microbiome may affect the embryo methylome and transcriptome (Pan et al. 2018). The exact mechanism has not yet been clarified. However, it can be assumed that the sperm microbiome interacts with the female microbiome in such a way that inflammatory cytokines are activated. Alternatively, the sperm microbiome releases various agents that promote apoptosis in the developing embryo (Calogero et al. 2017). Besides, microbial exchanges between partners promote the modification of the microbiome of each partner, and both of them influence the developmental potential of the embryo. To the best of our knowledge, the microbiome affects male fertility directly, which in turn affects the developing embryo indirectly. Moreover, positive vaginal and seminal cultures resulted in lower implantation rates and an increased percentage of miscarriages (Wittemer et al. 2004).

The presence of viruses in the microbiome is not consistent with the symbiosis theory, as presented previously. Viruses either make their cycle by abandoning their host cell or by intercalating the DNA of their host cell and regenerating as a part of the host cell's DNA. In the latter case, the virus and the host cell cohabitate, and in this case, symbiosis cannot be as useful as in the previous presence of microbiota. Chronic virus infection is negatively associated with male fertility, but the chances of pregnancy success are not compromised. In the case of HCV, HBV, and HIV infections where the presence of viruses has been documented in seminal fluid but not in spermatozoa themselves (except for the HBV infection), routine semen processing with good practice in an IVF laboratory should be used when performing assisted reproduction in men tested positive for the above viruses (ESHRE Guidelines 2021). The presence of HBV infection in males seems not to affect the IVF outcome (Zheng et al. 2016; Cito et al. 2021). There are contradictory results regarding the effects of male HCV infection on infertility treatment outcomes. Although the fertilization rate has been reported to be significantly lower in couples

with HCV-RNA-positive men, other studies report that HCV infection does not affect the IVF-ICSI cycle outcomes in these couples (Yang et al. 2015; Cito et al. 2021; ESHRE Guidelines 2021). Couples with a male partner testing positive for HIV-1 should be informed that the efficacy of ART is not affected compared to HIV-seronegative couples (Marques et al. 2015; ESHRE Guidelines 2021). As far as HPV is concerned, ART outcomes are affected by the presence of HPV in male partners (Depuydt et al. 2019; Garolla et al. 2016). Much attention should be given to IUI programs, since the HPV testing of couples undergoing ART treatment is not included in the European Tissue and Cells Directive. Testing for HPV is challenging and appears to be mandatory (Anagnostou et al. 2023, under review), and its importance still needs to be clarified in the field of infertility treatment and assisted reproduction. Finally, there are no studies regarding the effect of SARS-CoV-2 infection on assisted reproduction. According to the recommendations of international societies, couples with acute infections are strongly advised not to attempt IVF until they fully recover from the illness. Nevertheless, recent studies have found no effect on IVF outcomes (Albeitawi et al. 2022; Kabalkin et al. 2022).

5.4 Therapeutic Approaches for Male Infertility

In this section, potential treatment approaches for male infertility associated with microbial dysbiosis will be highlighted. As far as the viral infection is concerned, the sole therapeutic approach for virus infection is antiviral therapy in order to eliminate the activation of the virus during medical assisted reproduction (MAR) treatments, offering extra caution in the case of possible vertical transmission. Protection measurements for any possible virus transmission have been recommended (ESHRE guidelines).

With regard to microbial infections, the therapeutic approach has to do with whether the infection is asymptomatic or a recurrent and latent infection. In the first case, antibiotic therapy seems to be beneficial in maintaining or restoring sperm parameters in infertile patients (Farsimadan and Motamedifar 2020). Antibiotic treatment for *Mycoplasma hominis* and *C. trachomatis* infections resulted in increased chances of getting spontaneously pregnant (Ahmadi et al. 2017, 2018). However, antibiotic therapy appears to have deleterious effects on sperm parameters in a number of cases (Calogero et al. 2017), although it seems that is the only therapeutic approach to prevent or control asymptomatic infections. Indeed, antibiotic therapy may serve as a cornerstone for treating several microbial infections, but it should be applied with extra caution so as to treat male infertility and not damage sperm parameters. The balance between benefits and harm has not yet been evaluated for the use of antibiotics for male infertility treatment.

In the case of recurrent microbial infections, antibiotic therapy appears not to have any positive effects. It is possible that microbial symbiosis has settled down and that a few days of antibiotic therapy are not enough to overcome the almost permanent negative effect of the microbiome on sperm parameters. An alternative therapeutic approach in such cases is probiotic supplementation to manage male

infertility. Oral supplementation of probiotics in order to overcome the gut microbiota has been documented to improve all sperm parameters, including elevation of progressive motility and reduction of sperm DNA fragmentation and oxidative stress. Similarly, the administration of probiotics in infertile men proved beneficial, since it resulted in improved semen volume, concentration, and progressive motility (Maretti and Cavallini 2017). Recently, intervention with *Lactobacillus* and *Bifidobacteria* species in infertile men led to a significant reduction in inflammatory markers while documenting a significant increase in sperm ejaculate, total sperm count, and progressive motility (Helli et al. 2022). The protective effect of probiotics is likely to be due to the reduction of oxidative stress (Barbonetti et al. 2011). The role of probiotics and whether there is an age- or dose-dependent effect of probiotics on male fertility have not yet been clarified due to a limited number of studies. Future studies regarding the investigation of the mechanism underlying the effect of probiotics on sperm parameters may gain a clear understanding of the possible role of probiotics in male fertility. Several omics methods may help in investigating this issue (Farahani et al. 2021). Recent studies showed that the supplementation of *L. salivarius* PS11610 for a 6-month period with 1 dose/24 h for male patients significantly improved the microbiome of the urogenital tract and increased pregnancy chances (Iniesta et al. 2022). Alternatively, symbiotic administration (FamiLact) appears to enhance sperm quality, including chromatin status (Abbasi et al. 2021). A recent systematic review concluded that probiotic therapy improves sperm parameters, but due to the controversial results observed regarding probiotic therapy in women, high-quality studies are needed to examine if such treatment has any benefits over assisted reproduction (Corbett et al. 2021). Similar results have been observed in several animal models, including roosters, murines, and zebrafish (Mazanko et al. 2018; Valcarce et al. 2019; Keshtmand et al. 2021). The imbalance of the microbiome may cause a number of reproductive disorders, including infertility, compromising couples' IVF attempts. The administration of antibiotics during ART treatments has been widely used as prophylaxis for potential infections. However, the most common time for antimicrobial prophylaxis is at the time of embryo transfer, since it is possible that microbiota coming from the upper genital tract will grow in the transfer catheter tip and affect pregnancy outcome. A Cochrane review demonstrated that the administration of antibiotics during embryo transfer does not have any significant positive impact on the ART outcome (Kroon et al. 2012).

5.5 Conclusions and Future Perspective

Accumulating evidence has shown a direct association between semen dysbiosis and male infertility (Farahani et al. 2021; Wang et al. 2022; Alqawasmeh et al. 2022; Morawiec et al. 2022). Although research in this field is limited, the growing interest of researchers has caught much attention. A number of studies are focused on the mechanism by which the microbiome exerts its action on sperm parameters. For this purpose, inside the quiver, there are methods for the calculation of metabolomics and

transcriptomics that explore the mechanistic action of the microbiome on sperm parameters. Currently, next-generation sequencing provides specific insights into the composition of the seminal microbiome. There are studies investigating the role of antibiotics as well as the supplementation role of probiotics as a solution to the treatment of microbiome-related male infertility. Antibiotics may serve as a temporary solution for asymptomatic infected patients. A more attractive solution with promising results in chronically infected patients appears to be the oral supplementation of probiotics (Corbett et al. 2021; Farahani et al. 2021). Although there is no consensus regarding the long-term effects of probiotics, there is a trend toward their use as supplements.

The negative cultural status seems not to be enough to explain the poor sperm parameters. The symbiosis theory may serve several times in a beneficial manner, but when symbiosis is modified to dysbiosis, sperm parameters are impaired. Therefore, it is crucial to investigate the microbiome balance between symbiosis and dysbiosis. Although it is difficult to explore this delicate balance because each patient has a different (either in concentration or in regard to species) microbiome, next-generation sequencing may provide extra information for one of the main causes of male infertility. Upon further in-depth investigations regarding the action of the microbiome on male fertility, scientists may come closer to resolving the puzzle of male infertility. As far as viruses (in terms of the microbiome) are concerned, antiviral therapy for infected patients is the main choice of treatment. Although most viruses symbiotically live with human host cells, the long-term effects of this symbiosis seem to impair sperm parameters.

The microbiome, as innocent as it appears, has recently triggered investigations in order to resolve one of the oldest causes of male infertility. Updated molecular tools have provided insights into the role of all seminal flora. The use of these diagnostic tools may provide a deeper understanding of the action of the microbiome, while the testing of the microbiome for male infertility evaluation seems necessary.

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Genetic Testing in Polycystic Ovary Syndrome

6

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Abstract

Polycystic ovarian syndrome is the most common cause of anovulatory infertility. Familial aggregation, inheritance patterns, GWAS studies, and candidate gene studies have shown that PCOS is a complex, multifaceted disorder with a significant contribution from genetic factors. PCOS genetics has been investigated by 52 case-control and 8 GWAS studies. $(TTTTA)_n$ polymorphic repeat, rs4077582 C>T, rs11632698 A>G, and rs4887139 G>A polymorphisms in the CYP11A gene, -344C>T in the CYP11B gene, rs2414096 A>G in the CYP19 gene, and rs2293275 and rs13405728 in the LHCGR gene significantly affect the risk of PCOS. These genes/polymorphisms are strong candidates for inclusion in genetic panels for testing PCOS risk or evaluate cases genetically. The data on other polymorphisms, such as rs743572 T>C (CYP17), V281L G>T (CYP21), rs12470652 (LHCGR), rs2975760 and rs3792267 in the CAPN10 gene, and Gly972Arg C>T in the IRS-1 gene, are equivocal. Polymorphisms, including rs11632698 (CYP11A), CAG repeats in the AR gene, rs4539842, rs12470652, rs13429458, and rs2479106 in the LHCGR gene, rs3842570, rs5030952, rs7607759, rs2975766, rs2953152, rs2975762 in the CAPN10 gene, and Ala513Pro in the IRS-1 gene, do not affect PCOS risk. A total of eight GWAS studies have been conducted, and genetic polymorphisms in the *THADA (rs13429458)*, *DENND1A (rs2479106)*,

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LHCGR (rs13405728), *c9orf3 (rs4385527)*, *YAP1 (rs1894116)*, *RAB5B/SUOX (rs705702)*, *HMGA2 (rs2272046)*, *TOX3 (rs4784165)*, *INSR (rs2059807)*, *SUMO1P1 (rs6022786)*, *FSHR (rs2268363)*, *GYS2 (rs10841843*, *rs6487237*, *rs7485509)*, *GATA4/NEIL2 (rs804279)*, *c9orf3/FANCC (rs10993397)*, and *FSHB/ARL14EP (rs11031006)* genes were found to affect PCOS risk. Across these GWAS studies, *THADA*, *DENND1A*, and *TOX3* were common genes, making them strong candidates for case-control studies and possible inclusion in genetic testing in the future. At present, there is no genetic testing panel for PCOS, and genetic screening in PCOS cases is not practiced in clinics. Looking at the strong involvement of genetic factors and the polygenic nature of the disorder, the inclusion of genetic analysis in PCOS care would help in personalized medicine in the future.

Keywords

PCOS · Polycystic ovary syndrome · Genetic testing · Reproductive medicine · Genetic of PCOS

6.1 Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder in females, especially in women of reproductive age (Panda et al. 2016). The global prevalence of PCOS is estimated to be around 5–10% (Azziz 2016). The ailment is identified by the presentation of chronic anovulation and hyperandrogenism in females (Azziz 2016). The major etiological factors associated with PCOS include stress, obesity, and hormonal instabilities (Huddleston 2010). This condition is also referred to as Stein-Leventhal syndrome, which was named by American gynecologists Irving F Stein, Sr. and Michael L. Leventhal (Legro 2009). The revised Rotterdam criteria are the universally accepted criteria for the diagnosis of PCOS (ESHRE and Group. 2004), which require the exclusion of other causes of hyperandrogenism, like adrenal or pituitary dysfunction, and the occurrence of two of the following three characteristics: oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasound (Hiam et al. 2019).

The etiology of this syndrome is still notional, while its pathophysiology emerges to be both multifactorial and polygenic (Sheykhha et al. 2007) (Fig. 6.1). Candidate genes, gene-gene interactions, and gene-environment interactions have been reported to influence the predisposition to PCOS. Numerous studies have described the significance of genetic factors in the development of PCOS; however, no consensus has been reached regarding the identification of an established genetic marker for PCOS (Khan et al. 2019). Identifying the pathogenic mutations may help in delineating the genetic architecture of this complex, multifactorial disorder (Khan et al. 2019). There are two complementary types of genetic approaches that have been used most often for PCOS: genome-wide association studies (GWAS) and candidate gene studies.

Eight GWAS studies have been performed, and more than 52 candidate gene studies have been conducted to ascertain the clinical relevance of the genetic polymorphism in PCOS (Hiam et al. 2019).

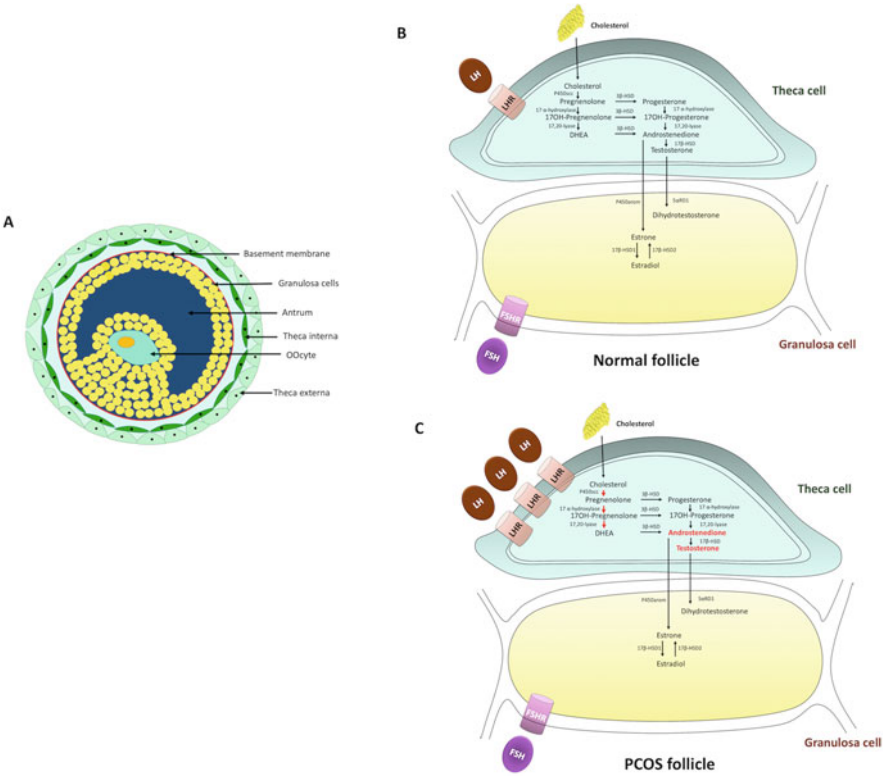


Fig. 6.1 (**a**) Histological architecture of an ovarian follicle. (**b**) Normal ovarian steroidogenesis. (**c**) Steroidogenesis in PCOS ovary. Compared to normal theca cells, PCOS theca cells show increased expression of *CYP17A1* gene, resulting in increased 17α-hydroxylase and 17,20-lyase activity, leading to enhanced androgen synthesis

6.2 Inheritance of PCOS

The genetic origin of PCOS was first described by Cooper and colleagues in 1968 (Cooper et al. 1968). Since then, the involvement of genetic factors in the etiology of PCOS has been thoroughly studied. Furthermore, its identification as a familial condition has long been noted (Prapas et al. 2009). The mode of inheritance of PCOS remains unknown to date. A few clinical genetic studies have reported that it follows an autosomal dominant mode of inheritance (Cooper et al. 1968; Wilroy Jr et al. 1975; Menke 2007), while others indicate the syndrome is likely a complex trait with an oligogenic basis (Jahanfar and Eden 1996; Franks et al. 1997). Nevertheless, recent studies suggest that this disorder is a complex trait that involves multifaceted interactions between multiple genes and environmental factors (Sheykhha et al. 2007). While the clustered cases among families strongly highlight

the genetic inheritance of the disease, the heterogeneous nature of the phenotype within the same family and in different families further suggests the significance of environmental factors. Vink et al. (2006) reported that the estimated variance in the pathogenesis of PCOS is 72% due to genetic influences (Vink et al. 2006).

6.3 Genetic Predisposition to PCOS

6.3.1 Candidate Gene Studies

PCOS shows a strong genetic etiology. All the genes and genetic variations that affect ovarian function directly or indirectly may affect PCOS risk. Some of the genes that are reported to affect PCOS risk include *CYP11A*, *CYP11B2*, *CYP17A1*, *CYP19*, *CYP21*, *AR*, *SHBG*, *FSHR*, *INSR*, *CAPN10*, *FTO*, and *FSHR*.

6.3.1.1 CYP11A

The CYP11A (15q24.1) gene encodes monooxygenase, which has an important role in the synthesis of androgens. The latter have been reportedly involved in the pathogenesis of polycystic ovary syndrome (PCOS). A $(TTTTA)_n$ microsatellite repeat expansion polymorphism in the promoter region of the CYP11A gene shows a protective effect against PCOS (Wang et al. 2006). A study on 201 cases and 147 controls in East Asian women suggested its possible role in the pathogenesis of PCOS (Wang et al. 2006) (Table 6.1). In 2012, another study on 314 PCOS patients and 314 controls targeting SNPs rs4077582 and rs11632698 in CYP11A showed a significant association of rs4077582 (C>T) with PCOS (Zhang et al. 2012b). Testosterone levels among the three genotypes of rs4077582 were different than in the control group, as were the LH levels and the LH/FSH ratio (Zhang et al. 2012b).

Table 6.1 CYP11A polymorphisms and their associations with PCOS

	Population	Sample size	*CYP11A* polymorphisms investigated	Association with PCOS	References
1.	East Asian (Chinese)	201 cases / 147 controls	$(TTTTA)_n$ microsatellite	$(TTTTA)_n$ repeat expansion have protective effect	Wang et al. (2006)
2.	East Asian (Chinese)	314 cases / 314 controls	rs4077582/ rs11632698	rs4077582 C>T substitution increases the risk	Zhang et al. (2012b)
3.	South Indian	267 cases/ 275 controls	$(TTTTA)_n$ microsatellite	$(TTTTA)_n$ repeat size of >8 increases the risk	Reddy et al. (2014)
4.	North Indian	270 cases / 270 controls	rs4077582/ rs11632698/ rs4887139	rs4077582 C>T/ rs11632698 A>G/ rs4887139 G>A substitution increases the risk	Kaur et al. (2018)

Reddy et al. (2014), upon a study of 267 cases and 275 controls in a South Indian population, found $(TTTTA)_n$ microsatellite polymorphism of >8 repeats to increase PCOS risk and confirmed CYP11A to be a potential biomarker for PCOS (Reddy et al. 2014). In a recent study on 270 Indian PCOS cases and 27s0 controls, the analyses of rs11632698, rs4077582, and rs4887139 polymorphisms in the CYP11A gene showed significant associations of all three polymorphisms with PCOS (Kaur et al. 2018). The above studies taken together suggest that the rs4077582, rs11632698, rs4887139, and $(TTTTA)_n$ microsatellite repeat polymorphism in the CYP11A gene affect PCOS risk.

6.3.1.2 CYP11B

The relationship of *CYP11B* (8q24.3) with PCOS has not been extensively studied, except in a small Chinese study that analyzed this polymorphism in 92 individuals (Zhao et al. 2003). The frequencies of TC and CC variants at the −344T *CYP11B* locus were higher in PCOS cases. The levels of PRA, PANG 2, aldosterone, and testosterone were upregulated in individuals with -344CC variants in comparison to those with -344TT genotype and normal women. This study suggested that (T>C) substitution at the -344 locus of the CYP11B gene increases the risk of PCOS (Zhao et al. 2003). However, there was no further study analyzing this polymorphism in PCOS.

6.3.1.3 CYP17

CYP17 (10q24.3) encodes the enzyme 17-alpha-hydroxylase, a regulatory factor in hyperandrogenemia (Qin and Rosenfield, 1998). This enzyme converts pregnenolone to 17-hydroxypregnenolone and progesterone to 17-hydroxyprogesterone, which is a rate-limiting step in androgen biosynthesis (Feigelson et al. 1998). Only one polymorphism (rs743572) in the gene has been analyzed in PCOS (Table 6.2). A study on 81 Caucasian women suggested a significant association between rs743572 and PCOS (Carey et al. 1994). Pulsalkar et al. concluded that *CYP17* T>C substitution (rs743572) increased PCOS risk (Pusalkar et al. 2009). Similarly, Kaur et al. analyzed 250 cases and 250 controls and observed a significant association between rs743572 and PCOS in a mixed Indian population from India (Kaur et al. 2018). In the Caucasian population of Iran, a study showed a significantly higher frequency of *CYP17* TC genotype in patients compared to controls (Rahimi et al. 2019), whereas in Pakistani women, a similar study suggested a significant association of *CYP17* 5'-UTR MSPA1 (rs743572) polymorphism with PCOS (Munawar Lone et al. 2021). All these studies, along with several others, present a strong case for the involvement of the *CYP17A1* genetic variation (rs743572) in PCOS.

Nevertheless, almost an equal number of studies reported no association between *CYP17A1* rs743572 and PCOS. For example, a study by Chua et al. reported no association of *CYP17A1* polymorphism with PCOS (Chua et al. 2012). No such association between CYP17A1 polymorphism and PCOS was found in polish population as well (Marszalek et al. 2001) Similarly, rs743572 or any other variation in this gene did not show an association with PCOS in Indians (Dasgupta et al. 2012) or

Table 6.2 CYP17 gene polymorphism and their association with PCOS

	Ethnicity	Sample size	CYP17 SNP investigated	Association with PCOS	References
1.	Caucasian	81 cases	rs743572	rs743572 T>C substitution increases the risk	Carey et al. (1994)
2.	Polish	55 cases/ 56 controls	rs743572	rs743572 T>C substitution does not increase the risk	Marszalek et al. (2001)
3.	American	195 cases	rs743572	rs743572 T>C substitution does not increase the risk	Kahsar-Miller et al. (2004)
4.	Indian	100 cases/ 100 controls	rs743572	rs743572 T>C substitution increases the risk	Pusalkar et al. (2009)
5.	Caucasian	287 cases/ 187 controls	rs743572	rs743572 T>C substitution does not increase the risk	Chua et al. (2012)
6.	Indian	60 cases/ 54 controls	rs743572	rs743572 T>C substitution does not increase the risk	Dasgupta et al. (2012)
7.	Indian	250 cases/ 250 controls	rs743572	rs743572 T>C substitution increases the risk	Kaur et al. (2018)
8.	Iranian/ Kurdish	50 cases/ 109 controls	rs743572	rs743572 T>C substitution increases the risk	Rahimi et al. (2019)
9.	Indian/ Kashmiri	394 cases/ 306 controls	rs743572	rs743572 T>C substitution increases the risk	Ashraf et al. (2021a)
10.	Pakistani	204 cases/ 100 controls	rs743572	rs743572 T>C substitution increases the risk	Munawar Lone et al. (2021)

Americans (Kahsar-Miller et al. 2004). In conclusion, it can be stated that the association of rs743572 with PCOS is equivocal.

6.3.1.4 CYP19

The *CYP19* gene (15p21.13) encodes aromatase, an enzyme that converts androgens to estrogens in gonadal and extragonadal tissues. In PCOS patients, aromatase activity was found to be decreased, which was independent of BMI (Chen et al. 2015). This decreased activity of aromatase leads to hyperandrogenism, which results in hormonal imbalance, infertility, and early puberty in young girls and women of reproductive age. In 186 cases and 71 controls from a Spanish population, rs2414096 (A>G) showed a significant association with PCOS (Petry et al. 2005). The distributions of aromatase haplotypes and aromatase SNP_50 (rs2414096) genotypes were significantly different between precocious pubarche (PP) girls and controls. This study suggested that common variations in the aromatase gene (and not just rare loss-of-function mutations) are associated with androgen excess in girls and young women (Petry et al. 2005). Genetic polymorphisms in the *CYP19* gene

Table 6.3 *CYP19* polymorphisms and their associations with PCOS

	Population	Sample size	*CYP19* SNP investigated	Association with PCOS	References
1.	Spanish	186 cases/ 71 controls	rs2414096 (SNP_50)	rs2414096 A>G substitution increases the risk	Petry et al. (2005)
2.	Chinese	364 cases/ 297 controls	rs2470152	rs2470152 T>C substitution does not increase the risk	Zhang et al. (2012a)
3.	Egyptian	30 cases/ 30 controls	rs2414096	rs2414096 A>G substitution increases the risk	Mostafa et al. 2016
4.	Iranian	70 cases/ 70 controls	rs2414096	rs2414096 A>G substitution increases the risk	Mehdizadeh et al. (2017)
5.	Pakistani	204 cases/ 100 controls	rs2414096	rs2414096 G>A substitution increases the risk	Munawar Lone et al. (2021)
6.	Indian/ Kashmiri	394 cases/ 306 controls	rs2414096	rs2414096 A>G substitution increases the risk	Ashraf et al. (2021b)

are associated with serum estradiol (E2) levels and the E2/T (estradiol/testosterone) ratio (Zhang et al. 2012a). A study on 364 Chinese cases and 297 controls revealed that rs2470152 in *CYP19* is not associated with the risk of PCOS; however, the heterozygous TC genotype may inhibit aromatase activity, resulting in hyperandrogenism (Zhang et al. 2012a). In Egyptian women, a study on 30 cases and 30 controls found that the genotype distribution at the rs2414096 (A/G) locus was significantly different in PCOS women as compared to controls, which relates to reduced aromatase activity and subsequent hyperandrogenism in PCOS Egyptian women (Mostafa et al. 2016).

Genetic analysis of 70 Iranian women with PCOS and 70 controls revealed a potential association between *CYP19* rs2414096 (A>G) and PCOS (Mehdizadeh et al. 2017). Similarly, a study on 204 Pakistani PCOS cases and 100 controls found a possible association of *CYP19* rs2414096 with PCOS risk (Munawar Lone et al. 2021). The association of the rs2414096 (A>G) polymorphism in the *CYP19* gene with PCOS was also observed in Kashmiri women with features of hyperandrogenism (Ashraf et al. 2021b) (Table 6.3).

Conclusively, all these studies suggested that PCOS patients have an androgen imbalance, obesity, an altered BMI, and, in some cases, thyroid issues as well. It can be concluded that *CYP19* polymorphisms affect the risk of PCOS, but no single polymorphism accounts for the risk in different populations. Yet, we can consider that *CYP19* SNP rs2414096 may be a suitable genetic marker for identifying predisposition to PCOS; other polymorphisms need to be analyzed in larger sample sizes across different ethnicities.

6.3.1.5 CYP21

CYP21 (6p21.33) encodes an enzyme called 21-hydroxylase, which is responsible for the conversion of 17-hydroxyprogesterone to cortisol and progesterone to aldosterone. In the case of a mutation in *CYP21*, a deficiency of 21-hydroxylase occurs, which leads to high androgen levels in affected females, which is one of the features of PCOS. These individuals also suffer from congenital adrenal hyperplasia (CAH), as the mutation renders the cells of the adrenal gland unable to produce cortisol and aldosterone. The most commonly identified mutation in *CYP21A2* is the V281L (G>T) substitution in exon 7 (Witchel and Aston 2000). A study on 30 adolescent girls with hyperandrogenism, 14 healthy control women, and 15 female obligate *CYP21* mutation carriers concluded that *CYP21* nonsense mutation carriers tend to be asymptomatic, while missense mutation carriers, i.e., V281L, appear to manifest a PCOS phenotype (Witchel and Aston 2000) (Table 6.4).

A study on 114 cases and 95 controls from a non-Hispanic white population with adrenal androgen excess reported that *CYP21* mutations seem to play a limited role in the development of PCOS (Witchel et al. 2005). In contrast, another study on 50 Italian women with PCOS and 60 controls analyzed the V281L polymorphism in PCOS and reported no association with PCOS (Pucci et al. 2010). While there have been limited studies on *CYP21* polymorphisms in PCOS, the available data is contradictory, suggesting no *CYP21* polymorphism is a great genetic marker for PCOS.

6.3.1.6 AR

Androgen receptor (Xq11-q12) encodes for nuclear receptor protein, to which the androgens bind, resulting in the development of secondary sexual characters and other related functions. This gene contains a highly polymorphic $(CAG)_n$ microsatellite polymorphism in its first exon. Variations in the number of triplet repeats have been analyzed in PCOS by a number of studies (Table 6.5). Analysis of the *AR*-CAG repeat in 106 Finnish women with PCOS and 112 controls reported no association between the two (Jääskeläinen et al. 2005). Similarly, the analysis of CAG repeats in 117 Slovene PCOS cases reported no association with PCOS (Ferk et al. 2008).

Table 6.4 *CYP21* polymorphisms and their associations with PCOS

	Population	Sample size	*CYP21* polymorphisms investigated	Association with PCOS	References
1.	American	30 cases/ 14 controls	V281L	V281L G>T substitution increases the risk	Witchel and Aston (2000)
2.	Non-Hispanic white	114 cases/95 controls	V281L	V281L G>T substitution have no significant association	Witchel et al. (2005)
3.	Italian	50 cases/ 60 controls	V281L	V281L G>T does not increase the risk	Pucci et al. (2010)

Table 6.5 *AR* CAG repeat polymorphism in PCOS

	Population	Sample size	*AR* SNP investigated	Association with PCOS	References
1.	Finnish	106 cases/ 112 controls	CAG repeats	CAG repeats have no significant association	Jääskeläinen et al. (2005)
2.	Slovene	117 cases	CAG repeats	CAG repeats have no significant association	Ferk et al. (2008)
3.	Croatian	214 cases/ 209 controls	CAG repeats	CAG repeats have no significant association	Akratic et al. (2012)
4.	Indian	169 cases/ 175 controls	CAG repeats	CAG repeats have no significant association	Rajender et al. (2013)
5.	Turkish	182 cases/ 172 controls	CAG repeats	CAG repeats ≤17. Increases the risk of PCOS	Polat et al. (2020)
6.	Iraqi	150 cases/ 100 controls	CAG repeats	CAG repeats have no significant association	Ramadhan et al. (2022)

Similar conclusions were drawn by a study analyzing *AR* CAG repeat in 214 Croatian women and 209 controls (Skrgatic et al. 2012). A study on Indian populations analyzed CAG repeat length in 169 cases and 175 controls and conducted a meta-analysis on 2194 cases and 2242 controls to conclude that CAG biallelic mean length was similar between cases and controls (Rajender et al. 2013). A study on 182 cases and 172 controls in the Turkish population found that the risk of PCOS is associated with shorter CAG repeats (Polat et al. 2020). Similarly, a total of 150 cases and 100 controls from Iraq were studied, and no association was observed between AR CAG repeats and PCOS (Ramadhan et al. 2022). From the above, it appears that AR-CAG repeats have no significant association with PCOS, and testing this has no clinical significance.

6.3.1.7 LHCGR

Luteinizing hormone and human chorionic gonadotropin both bind to a common receptor called LHCGR. Accordingly, *LHCGR* polymorphisms have been studied in PCOS. In women of Sardinian origin, three SNPs in the LHCGR gene were analyzed [ins18LQ rs4539842 in exon 1, N291S rs12470652, and S312N rs2293275 in exon 10] in 198 cases and 187 controls. This study found a strong association between the *LHCGR* S312N rs2293275 (G>A) substitution and PCOS (Capalbo et al. 2012) (Table 6.6). Similarly, a study on 100 Egyptian PCOS cases and 60 controls confirmed the association of SNP rs2293275 (G>A) (G935A) with PCOS (Bassiouny et al. 2014). In the southern region of India, a study on 204 cases and 204 controls reported an association of the *LHCGR* polymorphism rs2293275 with PCOS (Thathapudi et al. 2015). In a study on the Chinese population, 151 cases and 99 control women of Hui ethnicity were studied, and the rs13405728 polymorphism in *LHCGR* was found to be significantly associated with PCOS (Ha et al. 2015). Investigation on the possible association between rs1056917 (G1502A) polymorphism in the *LHb* gene and rs2293275 (G935A) and ins18LQ (rs4539842) in the *LHCGR* genes with PCOS in 210 Egyptian cases and 200 controls (El-Shal et al.

Table 6.6 *LHCGR* polymorphisms and their associations with PCOS

	Population	Sample size	*LHCGR* SNP investigated	Association with PCOS	References
1.	Sardinian	198 cases/ 187 controls	rs4539842/ rs12470652/ rs2293275	rs2293275 G>A increases the risk	Capalbo et al. (2012)
2.	Egyptian	100 cases/60 controls	rs2293275	rs2293275 G>A increases the risk	Bassiouny et al. (2014)
3.	South Indian	204 cases/ 204 controls	rs2293275	rs2293275 A>G increases the risk	Thathapudi et al. (2015)
4.	Chinese	151 cases/99 controls	rs13405728/ rs13429458/ rs2479106	rs13405728 C>T increases the risk	Ha et al. (2015)
5.	Egyptian	210 cases/ 200 controls	rs2293275/ rs4539842/ *LHβ*rs1056917	rs2293275 G>A/ *LHβ* rs1056917 T>C increases the risk	El-Shal et al. (2016)
6.	North Indian	150 cases/ 150 controls	*LHβ*rs1056917, *LHR*rs61996318	*LHβ* rs1056917 T>C/ *LHR*rs61996318 C>A increases the risk	Deswal et al. (2019)
7.	Jordanian	55 cases/ 55 controls	rs2293275	rs2293275 A>G increases the risk	Atoum et al. (2022)
8.	North Indian	421 cases/ 322 controls	rs2293275, rs12470652	rs2293275 G>A/ rs12470652 T>C increases the risk	Singh et al. (2022)

2016) found that rs1056917 (*LHb*) and rs2293275 (*LHCGR*) were strongly associated with PCOS, and interestingly both these polymorphisms create a synergistic effect when present together (El-Shal et al. 2016). Another study (Deswal et al. 2019) on North Indian women analyzed the association of *LHβ* (rs1056917) and lutropin receptor (*LHR*) (rs61996318) polymorphisms with PCOS in 150 cases and 150 controls. It was found that *LHβ* TC and CC genotypes and *LHR* CA and AA genotypes conferred a significant risk of PCOS (Deswal et al. 2019). In a Jordanian population, 55 cases and 55 controls were analyzed, and the study reported a statistically significant difference in the frequency of AG genotype at the rs2293275 locus between PCOS patients and controls (Atoum et al. 2022). Patients with GG and AG genotypes at this locus of *LHCGR* showed statistically significant higher LH and LH/FSH values compared to controls. *LHCGR* genotypes at the rs2293275 locus could modulate hormonal levels associated with hirsutism, oligomenorrhea, and BMI (Atoum et al. 2022). Another study from India on 421 cases and 322 controls, aimed to evaluate the association of LHCGR

rs2293275 G>A and rs12470652 T>C polymorphisms with PCOS, concluded that *LHCGR* plays a critical role in the progression of PCOS (Singh et al. 2022). Considering all the abovementioned investigations, it can be concluded that LHCGR polymorphisms alone or in combination with the LHβ gene polymorphisms affect the risk of PCOS significantly. Therefore, it is worth analyzing these genes for genetic polymorphisms in PCOS cases. Further studies on these genes may identify specific mutation hotspots that could be preferentially tested in PCOS cases.

6.3.1.8 CAPN10

The calpain-10 gene (2q37.3) is involved in insulin action and secretion. Women with PCOS typically suffer from insulin resistance and obesity; hence, the *CAPN10* gene may be linked with PCOS. A study on the Spanish population, including 55 PCOS cases and 93 healthy controls, investigated the UCSNP-44, 43, 19, and 63 polymorphisms and concluded that the UCSNP-44 allele is associated with PCOS in Spanish women (Gonzalez et al. 2002) (Table 6.7). Another consecutive study on 148 Spanish women with PCOS and 93 controls also confirmed the association of UCSNP-44 with PCOS (Gonzalez et al. 2003). Yet another study on 88 South Brazilian women with PCOS cases claimed that UCSNP-43 was associated with metabolic disorders in PCOS, whereas UCSNP-19 and UCSNP-63 were unrelated, suggesting that the UCSNP-43 polymorphism may influence the risk of PCOS (Wiltgen et al. 2007). A study on 50 Chilean women with PCOS and 70 controls analyzed 3 SNPs in the Calpain-10 gene (UCSNPs-43, 19, and 63) and observed a significant association of the rare allele (A) at the UCSNP-43 (intron 3) locus with an increased risk of PCOS, while UCSNP-63 and UCSNP-19 were not associated with PCOS (Márquez et al. 2008).

146 German PCOS women and 606 controls were studied for UCSNP-44 (rs2975760), −43 (rs3792267), −56 (rs2975762), ins/del-19 (rs3842570), −110 (rs7607759), −58 (rs2975766), −63 (rs5030952), and − 22 (rs2953152) (Vollmert et al. 2007). This study revealed that women carrying the AA genotype at UCSNP-56 (rs2975762) (intron 4) had a higher susceptibility to PCOS in comparison to those carrying the GG genotype. The 22-genotype (2 repeat allele) of the UCSNP-19 ins/del (rs3842570) (intron 6) variant, which is in high linkage disequilibrium with UCSNP-56, was also significantly associated with PCOS (Vollmert et al. 2007). A study on 164 Korean women with PCOS and 325 controls studied *CAPN10* UCSNP-43, 19, and 63 polymorphisms and confirmed the involvement of *CAPN10* UCSNP-43 (GA) in insulin resistance and PCOS (Lee et al. 2009). Another study on 250 Indian PCOS cases and 299 controls analyzed UCSNP-44, 43, 56, 19, and 63 polymorphisms and found a significant association of UCSNP-44 genotype *CC* with PCOS (Dasgupta et al. 2012). A study on 127 Tunisian women with PCOS and 150 controls analyzed UCSNP-43 (rs3792267), UCSNP-19 (rs3842570), and UCSNP-63 (rs5030952) in 127 PCOS cases and 150 controls and denied the association of SNPs of *CAPN10* gene variants with PCOS (Ben Salem et al. 2014).

CAPN10 gene polymorphisms have been studied in a number of populations, and most of the studies suggest their association with PCOS. While the type of

Table 6.7 *CAPN10* polymorphisms and their associations with PCOS

	Population	Sample size	*CAPN10* SNP investigated	Association with PCOS	References
1.	Spanish	55 cases/ 93 controls	rs2975760/rs3792267/ rs3842570/rs5030952	rs2975760 T>C substitution increases the risk	Gonzalez et al. (2002)
2.	Spanish	148 cases/93 controls	rs2975760/rs3792267/ rs3842570/rs5030952	rs2975760 T>C substitution increases the risk	Gonzalez et al. (2003)
3.	South Brazilian	88 cases (cross-sectional)	rs3792267/rs3842570/ rs5030952	rs3792267 G>A substitution increases the risk	Wiltgen et al. (2007)
4.	Caucasian	146 cases/ 606 controls	rs2975760/rs3792267/ rs2975762/rs3842570/ rs7607759/rs2975766/ rs5030952/rs2953152	rs2975762 G>A substitution/ rs3842570 (2 repeats) increases the risk	Vollmert et al. (2007)
5.	Chilean	50 cases/ 70 controls	rs3792267/rs3842570/ rs5030952	rs3792267 G>A substitution increases the risk	Márquez et al. (2008)
6.	Korean	210 cases/ 442 controls	rs3792267/rs3842570/ rs5030952	rs3792267 G>A substitution increases the risk	Lee et al. (2009)
7.	Indian	250 cases/ 299 controls	rs2975760/rs3792267/ rs2975762/rs3842570/ rs5030952	rs2975760 T>C substitution increases the risk	Dasgupta et al. (2012)
8.	Tunisian	127 cases/ 150 controls	rs3792267/rs3842570/ rs5030952	No risk associated with any of these SNPs	Ben Salem et al. (2014)

polymorphism and the level of association vary across populations, *CAPN10* appears to be a good candidate gene for screening in PCOS cases.

6.3.1.9 IRS-1

The insulin receptor substrate 1 gene (2q36.3) encodes a protein that is phosphorylated by insulin receptor tyrosine kinase. Mutations in this gene are associated with T2DM and susceptibility to insulin resistance. A study on 146 Chilean women with PCOS and 97 healthy controls found an association between the Gly972Arg (rs1801278) polymorphism in the *IRS1* gene and PCOS (Sir-Petermann et al. 2004) (Table 6.8). This study claimed that the Gly972Arg polymorphism is associated with a decrease in insulin secretion in healthy women, whereas in PCOS cases, this polymorphism interacts with obesity to influence insulin resistance and hence contributes to the pathogenesis of metabolic components of PCOS (Sir-Petermann et al. 2004). A study on Chinese women, including 120 cases and 40 controls, reported that the rs1799817 polymorphism in

Table 6.8 *IRS1* polymorphisms and their associations with PCOS

	Population	Sample size	*IRS-1* SNP investigated	Association with PCOS	References
1.	Chilean	146 cases/97 controls	Gly972Arg	C>T substitution increases the risk	Sir-Petermann et al. (2004)
2.	Chinese	120 cases/40 controls	Gly972Arg	C>T substitution Increases the risk	Chen et al. (2004)
3.	Spanish	103 cases/48 controls	Gly972Arg	No risk associated with this SNP	Villuendas et al. (2005)
4.	Taiwanese	47 cases/45 controls	Gly972Arg/ Ala513Pro	No association of either of the SNPs	Lin et al. (2006)
5.	Japanese	123 cases/ 380 controls	Gly972Arg	C>T substitution Increases the risk	Baba et al. (2007)
6.	Italian	65 cases/27 controls	Gly972Arg	C>T substitution Increases the risk	Pappalardo et al. (2010)
7.	Iranian	48 cases/52 controls	Gly972Arg	No risk associated with this SNP	Rashidi et al. (2012)
8.	Croatian	150 cases/ 175 controls	Gly972Arg	No risk associated with this SNP	Skrgatić et al. (2013)
9.	Indian	249 cases/ 100 controls	Gly972Arg	No risk associated with this SNP	Rasool et al. (2022)

exon 17 of the insulin receptor gene (*INSR*) increases susceptibility to PCOS, especially in nonobese patients (Chen et al. 2004). A study on 103 Spanish PCOS women and 48 controls found that Gly972Arg polymorphism in the IRS-1 gene and Gly1057Asp polymorphism in the *IRS-2* gene influence glucose homeostasis in premenopausal women but are not associated with PCOS (Villuendas et al. 2005).

Pappalardo et al. (2010), in a study on 65 cases and 27 controls, found *IRS-1* Gly972Arg (rs1801278) to be linked with PCOS in Italian women. On the other hand, *IRS-1* Gly972Arg and *IRS-2* Gly1057Asp polymorphisms were found to be unrelated to PCOS in Iranian women upon analysis of 48 PCOS cases and 52 controls (Rashidi et al. 2012). A study on Taiwanese women recruited 47 Taiwanese Hoklo and Hakka women with PCOS and 45 healthy Hoklo and Hakka women. They observed a high prevalence of abnormal glucose tolerance and insulin resistance, but no association of Gly972Arg and Ala513Pro polymorphisms with PCOS was observed (Lin et al. 2006). However, a study on Japanese women, including 123 cases and 380 controls, found that the *IRS-1* Gly972Arg polymorphism was significantly associated with PCOS in Japanese women (Baba et al. 2007). In a study on Croatian women, including 150 PCOS cases and 175 controls, no significant association between VNTRs *INS*, C/T *INSR*, and Gly792Arg *IRS-1* polymorphisms and PCOS was observed (Skrgatić et al. 2013). In India, a study on 249 Kashmiri women with PCOS and 100 controls observed no association between *IRS-1*Gly972Arg (rs1801278) (G>A) and PCOS (Rasool et al. 2022). *IRS-1* polymorphisms have been reported to pose PCOS risk in women of Chinese, Chilean, Italian, and Japanese ethnicities; on the other hand, they show no

association in Taiwanese, Iranian, Spanish, Croatian, and Indian women with PCOS. Hence, *IRS-1* may be a good candidate gene for analysis in certain ethnicities.

6.3.2 Genome-Wide Association Studies

Genome-wide association studies (GWAS) have evolved over the past two decades into a powerful approach for exploring the genetic architecture of complex human diseases. GWAS allows the detection of novel susceptibility loci associated with predisposition to diseases, including those loci that confer only modest risk. Since PCOS is a complex disease, GWAS on this subject have been conducted, providing certain leads in identifying the genetic basis of PCOS. A total of eight GWAS studies have been performed on PCOS (Fig. 6.2).

6.3.2.1 GWAS 1

The first GWAS on PCOS was conducted on Han Chinese population, which included a discovery set of 744 PCOS cases and 895 controls, followed by a replication set including 2 independent cohorts (2840 PCOS cases and 5012 controls from northern Han Chinese and 498 cases and 780 controls from southern and central Han Chinese). This study claimed strong evidence of PCOS susceptibility with loci 2p16.3 (rs13405728), 2p21 (rs13429458), and 9q33.3 (rs2479106), which mapped to three genes: *LHCGR* (luteinizing hormone/choriogonadotropin receptor), *THADA* (THADA armadillo repeat containing), and *DENND1A* (DENN domain containing 1A), respectively (Chen et al. 2011).

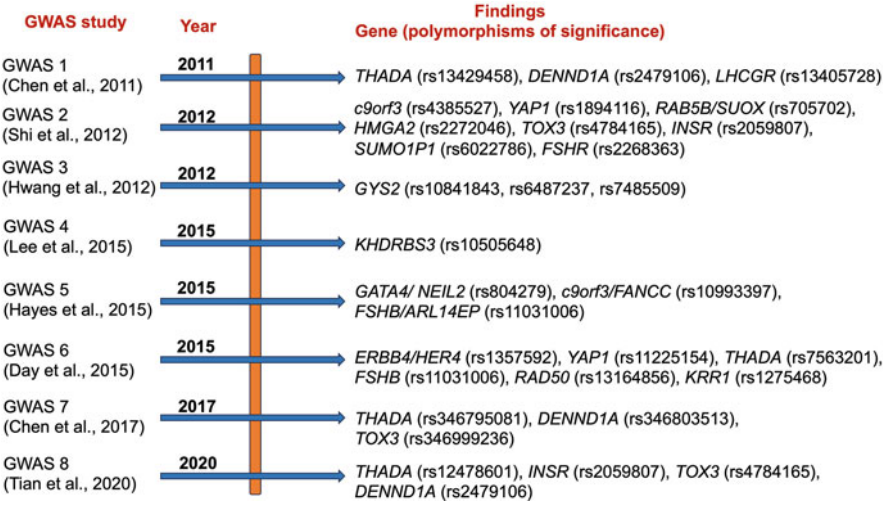


Fig. 6.2 GWAS studies in chronological order

6.3.2.2 GWAS 2

Another GWAS (GWAS 2) by Shi et al. (2012), which was a follow-up to the previous GWAS (GWAS 1), found eight new signals in addition to the previously identified three loci. The eight new PCOS association signals were mapped to 9q22.32 (rs4385527, *C9orf3*), 11q22.1 (rs1894116, *YAP1*), 12q13.2 (rs705702, *RAB5B/SUOX*), 12q14.3 (rs2272046, *HMGA2*), 16q12.1 (rs4784165, *TOX3*), 19p13.3 (rs2059807, *INSR*), 20q13.2 (rs6022786, *SUMO1P1*), and 2p16.3 (*FSHR* gene rs2268363). These PCOS association studies show evidence that candidate genes are related to insulin signaling, hormone function, and type 2 diabetes (T2D) in addition to calcium signaling and endocytosis (Shi et al. 2012).

6.3.2.3 GWAS 3

A genome-wide association study of PCOS (Hwang et al. 2012) in a Korean population (774 cases/967 controls) revealed *GYS2* (glycogen synthase 2) gene variation rs10841843 (C>T), rs6487237 (A>C), and rs7485509 (T>C) at 12p12.2 to affect PCOS risk. This study also found 14 other SNPs in the GYS2 gene (rs2417995, rs10841842, rs10492118, rs1871130, rs1871129, rs1871128, rs1904121, rs6487236, rs6487238, rs7962754, rs1580300, rs2126885, rs7485489, rs21697450) to be important. They did an association analysis with an adjustment for BMI and found that the signals are associated with PCOS and not just BMI-driven (Hwang et al. 2012).

Further case-control studies by Lerchbaum et al. (545 cases/317 controls), Goodarzi et al. (cohort A: 939 cases/957 controls; cohort B: 535 cases/845 controls), and Eriksen et al. (261 cases/248 controls) confirmed the association of the *DENND1A* and *THDA* gene variants with PCOS risk, in addition to hyperandrogenism and altered lipid profiles in women with PCOS (Lerchbaum et al. 2011; Goodarzi et al. 2012; Eriksen et al. 2013).

A study on the Han Chinese population in 2013 analyzed the earlier studied variants, which included *THADA* (rs13429458, rs12478601), *DENND1A* (rs2479106, rs10818854), and *LHCGR* (rs13405728) polymorphisms. This GWAS included 1731 PCOS cases and 4964 controls. The study found that variants rs13429458 C>A (*THADA*), rs12478601 T>C (THADA), and rs2479106 G>A (*DENND1A*) were significantly associated with the pathogenesis of PCOS in Han Chinese women (Cui et al. 2013).

Subsequently, the cross-ethnic effects of PCOS loci (*LHCGR*, *THADA*, *DENND1A*, *FSHR*, *c9orf3*, *YAP1*, *RAB5B/SUOX*, *HMGA2*, *TOX3*, *INSR*, and *SUMO1P1*) were studied in patients of Northern European descent. For this, a meta-analysis was performed, including an original study on a Dutch population (703 cases/2164 controls) combined with the results of previously published studies on PCOS patients from China (n = 2254) and the United States (n = 2618). Meta-analysis of the Dutch, US, and Chinese data displayed an association of PCOS with 12 significant variants: *LHCGR* rs13405728; *THADA* rs12468394 and rs12478601; *DENND1A* rs10818854 and rs10986105; *FSHR* rs2268361 and rs2349415; *c9orf3* rs4385527 and rs3802457; *YAP1* rs1894116; *RAB5B/SUOX* rs705702; and *SUMO1P1* rs6022786 (Louwers et al. 2013).

6.3.2.4 GWAS 4

In 2015, a GWAS on Korean women was conducted. It was done in two stages, where the first stage consisted of 976 cases and 946 controls and the second stage (replication study) had 249 cases and 778 controls. The study identified a novel susceptibility locus for PCOS on chromosome 8q24.2 polymorphism rs10505648, which was located upstream of the KHDRBS3 gene, possibly affecting PCOS risk, along with seven moderately associated loci at 4q35.2, 16p13.3, 4p12, 3q26.33, 9q21.32, 11p13, and 1p22 (Lee et al. 2015).

6.3.2.5 GWAS 5

In the same year, another GWAS was conducted on women of European ancestry (Hayes et al. 2015). This GWAS was done in multiple stages: Stage 1 involved 984 cases and 2964 controls; stage 2 included 1799 cases and 1231 controls; and after meta-analysis of the first and second studies, stage 3 consisted of 217 cases and 1335 controls. This study identified three cytogenetic loci at 8p32.1 (*GATA4/NEIL2*, rs804279), 9q22.32 (*c9orf3/FANCC*, rs10993397), and 11p14.1 (*FSHB/ARL14EP*, rs11031006) as susceptibility loci. Variants mapping to chr 8p32.1 and chr 11p14.1 were novel, while the one on chr 9q22.32 was previously reported in association with PCOS in Chinese women (Hayes et al. 2015).

6.3.2.6 GWAS 6

Day et al. (2015) performed a genome-wide association study on PCOS in 5184 cases of White European ancestry and 82,759 controls, with follow-up in a further ~2000 clinically confirmed cases and around ~100,000 controls. They identified six signals for PCOS at genome-wide statistical significance level in or near the genes *ERBB4/HER4* (rs1351592), *YAP1* (rs11225154), *THADA* (rs7563201), *FSHB* (rs11031006), *RAD50 (rs13164856)*, and *KRR1* (rs1275468) (Day et al. 2015).

6.3.2.7 GWAS 7

Chen et al. (2017) performed another GWAS on Chinese women. This study was conducted in two stages; the first stage included 200 PCOS cases and 228 controls, while the second stage used 200 more cases and 1400 controls in total. By primary selection and secondary verification at two stages in the experiment, three polymorphic sites, the *THADA* (rs346795081), *DENND1A* (rs346803513), and *TOX3* (rs346999236) genes, were found to contain significantly different allele frequencies between the patient and control groups. The allele frequencies at the three discovered polymorphic sites were significantly different between the patient and control groups, indicating their correlation with PCOS and the possible role of their corresponding genes in the pathogenesis of PCOS (Chen et al. 2017).

Later, Day et al. (2018) executed a meta-analysis on 10,074 PCOS cases and 103,164 controls of European ancestry. They identified 3 novel loci corresponding to the *PLGRKT* (rs10739076), *ZBTB16* (rs1784692), and *MAPRE1* (rs853854) genes and 11 previously reported loci, viz., *THDA* (rs7563201), *ERBB4* (rs2178575), *IRF1/RAD50* (rs13164856), *GATA4/NEIL2* (rs804279), *FANCC* (rs7864171), *DENND1A* (rs9696009), *ARL14EP/FSHB* (rs11031005), *YAP1* (rs11225154),

ERBB3/RAB5B (rs2271194), *KRR1* (*rs1795379*), and *TOX3* (rs8043701) to be associated with PCOS (Day et al. 2018).

6.3.2.8 GWAS 8

Another recent GWAS on 2082 Han Chinese cases identified 15 independent polymorphisms mapping to 11 GWAS loci (Tian et al. 2020). The CC group for rs12478601 in the *THADA* gene was associated with a decreased rate of metabolic syndrome (MS). The GG+AG group for rs2059807 in *INSR* was associated with an increased risk of MS. The GG+GT group for rs4784165 in *TOX3* was found to be associated with an increased rate of insulin resistance (IR). The GG+AG group for rs2479106 in *DENND1A* was associated with a decreased rate of IR. After the exclusion of PCOS cases with a family history of diabetes, hypertension, or dyslipidemia, the phenotype-genotype correlations between the genes *INSR* and *TOX3* and MS or IR were still significant. Three SNPs (rs13429458 in *THADA*, rs10818854 in *DENND1A*, and rs2059807 in *INSR*) were significantly associated with IR; however, their association was not significant after adjustment for age and BMI. This genotype-phenotype study thus provides clues that the *THADA*, *INSR*, *TOX3*, and *DENND1A* genes play roles in PCOS, possibly through a metabolic disorder-related pathway (Tian et al. 2020).

6.4 Genetic Testing in PCOS in Clinical Settings

At present, no genetic testing panel for PCOS is available. GWAS and genetic polymorphism studies on PCOS have clearly demonstrated its polygenic nature. Multigenic sequencing panels or several genetic polymorphism-based panels could be used for genetic analysis in PCOS cases. The ideal candidates for inclusion in such panels are $(TTTTA)_n$, rs4077582 C>T, rs11632698 A>G, and rs4887139 G>A polymorphisms in the CYP11A gene, -344C>T in the CYP11B gene, rs2414096 A>G in the CYP19 gene, and rs2293275 and rs13405728 in the LHCGR gene. Other genetic polymorphisms, such as rs743572 T>C (CYP17), V281L G>T (CYP21), rs12470652 (LHCGR), rs2975760 and rs3792267 in the CAPN10 gene, and Gly972Arg C>T in the IRS-1 gene, the data on which is equivocal, need further case-control studies and meta-analysis to clarify their suitability for inclusion in genetic testing panels. Other polymorphisms, including rs11632698 (CYP11A), CAG repeats in the androgen receptor, rs4539842, rs12470652, rs13429458, and rs2479106 in the LHCGR gene, rs3842570, rs5030952, rs7607759, rs2975766, rs2953152, rs2975762 in the CAPN10 gene, and Ala513Pro in the IRS-1 gene, appear unrelated to PCOS risk and are not worth pursuing further.

GWAS studies on PCOS have identified several other good candidates for analysis in case-control studies to assess their value as genetic markers. These include *rs13429458 in the THADA* gene, *rs2479106 in the DENND1A* gene, *rs13405728 in the LHCGR* gene, *rs4385527 in the c9orf3* gene, *rs1894116 in the YAP1* gene, *rs705702 in the RAB5B/SUOX* gene, *rs2272046 in the HMGA2* gene,

rs4784165 in the TOX3 gene, *rs2059807 in the INSR* gene, *rs6022786 in the SUMO1P1* gene, *rs2268363 in the FSHR* gene, *rs10841843*, *rs6487237*, *and rs7485509 in the GYS2* gene, *rs804279 in the GATA4/NEIL2* gene, *rs10993397 in the c9orf3/FANCC* gene, *and rs11031006 in the FSHB/ARL14EP* gene. Out of these, *THADA*, *DENND1A*, and *TOX3* appeared to be common across more than one GWAS study, making them strong candidates for case-control studies and possible inclusion in genetic testing panels in the future. At present, no genetic analysis panel for PCOS exists or is in use in clinics; however, the inclusion of genetic testing panels in PCOS care in clinics would help tailor treatment according to plausible genetic etiology. This presents a strong notion to design such panels for clinical settings.

6.5 Conclusions and Future Perspective

Familial aggregation, inheritance pattern, GWAS, and candidate gene studies have shown that PCOS is a complex, multifaceted disorder with a significant contribution from genetic factors. At present, no genetic screening panel for PCOS exists. Genetic investigation for the management of PCOS cases is not considered at present. In most of the cases diagnosed with PCOS, the focus is on treatment without any consideration of the possible etiology or the screening of genetic factors. The time for designing genetic panels for testing in PCOS is ripe, and the genes/polymorphisms listed above should be considered for inclusion in such tests. A total of 53 studies have been undertaken to assess the association between genetic polymorphisms and PCOS, and 8 GWAS studies have identified more than 15 strong candidate genes for analysis in PCOS. With a number of other candidate genes identified by GWAS studies, further case-control studies should preferentially focus on these genes to deeply dissect their association with PCOS across various ethnic populations. Genetic testing, once adopted in clinics, would help tailor the treatment of PCOS cases as per genetic and environmental factors unique to the cases under treatment.

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Genetic Testing in Premature Ovarian Failure

7

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Abstract

Premature ovarian failure is defined as the lack of ovarian hormones or ovarian follicle reserve. This results in aberrant ovarian activity, which causes early oocyte depletion and the start of menopause. Amenorrhea, a review of the family history going back at least three generations, a pelvic sonogram, and a serum FSH level $>$ 40 mIU/ml are all used to diagnose POF illness. The 2016 guidelines from the European Society of Human Reproduction and Embryology (ESHRE) recommended that amenorrhea be monitored for at least 4 months, increased FSH levels be tested for two consecutive months, and then karyotyping be used for genetic testing. 20–25% of the cases are believed to be genetic. A few genes have been discovered to affect the risk of POF, but 50–90% of the cases remain idiopathic. Since normal ovarian function is a complex process, many genes have yet to be discovered with a role in causing the disease. In this chapter, we have focused on genetic testing in POF cases. Different diagnostic methods, such as karyotyping, candidate gene sequencing, genome-wide association studies, whole genome/exome sequencing, array comparative genomic hybridization, and microarray, have been discussed in this chapter.

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Keywords

Premature ovarian failure · Genetic testing · Candidate gene sequencing · Microarray analysis · Next-generation sequencing · Genome-wide association study · Reproductive medicine

7.1 Introduction

Premature ovarian failure (POF), also known as premature ovarian insufficiency (POI), is a heterogenous disorder that affects 0.1% of women in their 20 s and 1–2% of women in their 30 s (Coulam et al. 1986). POF is a medical condition that is characterized by the deficiency of ovarian hormones or ovarian reserve, which leads to abnormal ovarian function, resulting in the early depletion of oocytes and the onset of menopause (Jankowska 2017; Wesevich et al., 2020). This results in abnormal reproductive cycles and infertility before the age of menopause. Patients suffering from POF present with a variety of features, such as a rise in gonadotropins, hypoestrogenism, amenorrhea, a decrease in functional follicles, an increase in the loss of follicles, and a menopausal level of follicle-stimulating hormone (FSH) and anti-Mullerian hormone (AMH) (Visser et al. 2012). However, in the long term, this disease is also followed by osteoporosis and cardiovascular or neurocognitive anomalies (Visser et al. 2012, Jankowska 2017). With regard to the etiology, POF is categorized into nongenetic (autoimmune, metabolic disorder, infection driven, or environmental causes) and genetic etiologies (Woad et al. 2006, Jiao et al. 2017, Rudnicka et al. 2018). Out of all causes of POF, nearly 20–25% are genetic, and of the genetic causes, 50–90% of cases are idiopathic despite being affected by first-degree relatives (Chapman et al. 2015, Qin et al. 2017, Cordts et al. 2011).

The diagnosis of POF disease is done through amenorrhea, family history analysis (at least for three generations), sonography of the pelvic region, and laboratory testing for serum FSH levels (>40 mIU/ml) (Nguyen et al. 2017). According to the European Society of Human Reproduction and Embryology (ESHRE) 2016 guidelines, POF diagnosis includes tracking for amenorrhea for at least 4 months, testing elevated FSH >25 IU/I for consecutive 2 months, and then genetic testing by karyotyping (Webber et al. 2016). The genetic causes of POF include several chromosomal abnormalities, genetic polymorphisms, and single-gene mutations (Qin et al. 2012). Earlier, the genetic causes of POF were investigated by analyzing genes that were directly or indirectly involved in folliculogenesis or ovarian function. Research using mouse models and the advent of sequencing technology made it easier to identify the candidate genes involved in POF, irrespective of their direct linkage to ovarian functions. Despite the large number of studies on the genetic etiology of POF identified (Table 7.1; Fig. 7.1), there are still a lot of conflicts and uncertainties in relating variants due to the unknown significance of either the polymorphism or the gene.

Table 7.1 Genes and their mutations associated with POF

Genes	Mutation	Full name	Function	Study type	Reference
AMH	G264R 790G > C; D288E 864C > G; R444H 1331G > A;	Anti-Mullerian hormone	Regulate synthesis of primary follicle by restricting follicular appointment by FSH	WES	Alvaro Mercadal et al. (2015)(4)
AMHR2	c.627 T > A; c.-482A > G	AMH receptor	Signal transduction of AMH	WES	Qin et al. (2014a) (2); Qin et al. (2015b)
ATM	c.2367C > G	Ataxia telangiectasia mutated	Phosphatidylinositol-3 kinase like protein kinase family member role in follicle synthesis	WES	Liu et al. (2020) (4)
BMP15	c. 510_512delT p.Y235C; p.R68W; p.A180T; p.L148P; c.181C > T, c.182G > A, c.985C > T, c.226C > T, c.227G > A, c.538G > A, c.538G > T, c.539C > T, c.617G > A, c.631C > T, c.661 T > C, c.727A > G, c. 202C > T, c. 413G > A, c.443 T > C; c. 588 T > A;	Bone morphogenetic protein 15	Follicular maturation through growth factor activity	WES	Di Pasquale et al. 2004(4); Kumar et al. (2017) (2); Dixit et al. (2006); Laissue et al. (2006) (3); Wang et al. (2010b); Rossetti et al. (2009); Patiño et al. (2017) (4);
BMPR2/ BMPR1A/ 1B	p.Arg442His; p.Phe272Leu	Bone morphogenetic protein receptor type2, 1A, 1B	Growth factor activity	ES	Renault et al. (2020) (4)
CLPP	c.433A > C; c.440G > C; c.270 + 4A > G	CIPP caseinolytic peptidase, ATP-dependent, proteolytic subunit homology	Maintenance of ovarian follicular reserve through its cytochrome -450 family 19 subfamily A activity	ES	Jenkinson et al. (2013) (4)
				WES	Qin et al. (2015a) (4)

(continued)

Table 7.1 (continued)

Genes	Mutation	Full name	Function	Study type	Reference
CSB-PGBD	c.643G > T c.3166G > A	CSB-PGBD3 fusion protein	Maintaining ovarian function through DNA damage repair		
CYP19A1	Intron: 15q21.1 (rs6493489) C > T; (rs6493488) C > G 3'UTR: (rs10046) T > C; (rs4646) C > A	Cytochrome P450, family 19, subfamily A, polypeptide 1	Regulate folliculogenesis through its cytochrome -450 family 19 subfamily A activity	CGS	Kim et al. (2011) (4)
eIF4ENIF1	c.2525A > C; c.1286C > G	Eukaryotic translation initiation factor 4E nuclear import factor 1	Germ cell development and regulate translation	GWAS	Zhao et al. (2019) (4); Qin et al. (2015b)
ERCC6	c.2444G > A; c.2510G > A	DNA excision repair protein ERCC-6	DNA mismatch repair	WES	Kuang et al. (2022); Jin et al. (2020)
ESR1	Intron: 6q25.1 (rs1569788) C > T; Intron: chr6:151842200 (rs2234693) T > C; c.-397C/T; c.-351A/G;	Estrogen receptor	Follicular growth and maturation	CGS	Kim et al. (2011) (4); Bretherick et al. (2008); M'Rabet et al. (2012); de Mattos et al. (2014) (3)
EXO1	Intron: chr1:241877473 (rs1635501) C > T; p. Thr52Ser;	Exonuclease 1	DNA mismatch repair	GWAS/ WES	Stolk et al. (2012) (2); Luo et al. (2020)
FANCA	c.1532 C > T; c. 1772G > A c. 3887A > G	Fanconi anemia, complementation group A	DNA repair protein	GWAS/ WES	Pyun et al. (2014) (4); Yang et al. (2019)
FIGLA	c.427G > C; c.252C > T; c. 419_421del ACA; c. 15-36del	Factor in the germ line alpha	Regulate genes of zona pellucida	WES	Tosh et al. (2015) (2); Zhao et al. (2008) (4)

FMR1	50-200CCG repeats	Fragile X Messenger Ribonucleoprotein 1	Development and maturation of oocyte	CGS	Oostra and Willemsen 2009(4); Dean et al. (2018) (2)
FOXL2	c.844_860dup17; c.898-927del; c.1009 T > A; c.13 T > A;	Forkhead box protein L2	Development of follicle and differentiation of granulosa cell	WES	Yang et al. (2017) (2); Harris et al. (2002); Bouilly et al. (2016)
FOXO3A	c.1262C > T; c. 1517G > A; c. 280C > T; c. 1021G > A; c. 1156C > T; c. 71C > A; c.140C > T; c.184G > A; c.1652C > T; c.1697C > G	Forkhead/winged helix box group O3	Regulates growth activation of primordial follicle	CGES	Watkins et al. (2006); Gallardo et al. (2008) (4); Wang et al. (2010a)
FSHR	c. 1222G > T; c. 1411A > T; c. 566C > T c. 1255G > A	Follicle-stimulating hormone receptor	Steroidogenesis of ovary and development of follicles	WES	Bramble et al. (2016) (2) Aittomäki et al. (1995); Layman et al. (1998); Da Fonte Kohek et al. (1998); Woad et al. (2013) (3); Doherty et al. (2002)
GDF9	c.199A > C; c. 646G > A; c. 147C > T; c. 557C > A; c. 712 A > G;	Growth differentiation factor 9	Granulosa cell proliferation, growth, and differentiation	CGES	Dixit et al. (2005); Laissue et al. (2006); Zhao et al. (2007);
HFM1	c. 3470G > A	ATP-dependent DNA helicase homolog	DNA replication for ovarian reserve	WES	Zhe et al. (2019) (4)
INHA	c. 769G > A;	Inhibin subunit alpha	Regulation of folliculogenesis via FSH inhibition	CGS	Shelling et al. (2000); Marozzi et al. (2002); Dixit et al. (2004) (3)

(continued)

Table 7.1 (continued)

Genes	Mutation	Full name	Function	Study type	Reference
KHDRBS1	c. 460A > G c. 263C > T	KH RNA-binding domain containing signal transduction-associated protein 1	mRNA translation and transport	WES	Wang et al. (2017)(4)
LHR	p. Leu608del; p. Val609del	Luteinizing hormone receptor	Ovulation, ovarian steroidogenesis, and maturation of follicle	CGES	Latronico et al. (1998); Persani et al. (2010) (3)
LHX8	c. 974C > T	LIM homeobox protein 8	Regulation of early oogenesis	CGES	Bouilly et al. (2016)
MCM8/9	c. 1732 + 2 T > C; c. 394C > T; c. 446C > G; c.1651C > T	Mini-chromosome maintenance component 8	Double strand break repair	WES	Wood-Trageser et al. (2014); AlAsiri et al. (2015); Desai et al. (2017) (4)
MSH4/5	c. 2355 + 1G > A; c. 1057C > A; c. 2107A > G; c. 1459G > T	MutS protein homolog 4/5	DNA mismatch repair	WES	Carlosama et al. (2017); Guo et al. (2017); Wang et al. 2020 (4)
NANOS3	c. 457C > T: c.358G > A	Nanos homolog 2	Germ cell development	CGES	Wu et al. 2013 (4); Santos et al. 2014 (2)
NBN	c. 651_661del5; c. 871C > T	Nibrin	DNA repair gene	NGS	Chrzanowska et al. (2010), Tucker et al. (2018) (4)
NOBOX	p. Arg355His; p. Arg303*; p. Arg117Trp; p. Thr190Hisfs*13; p. Lys371Thr; p. Arg449*; p. Asp452Asn;	Newborn ovary homeobox	Early folliculogenesis	WES	França et al. (2017) (2); Rajkovic et al. (2004) (4); Justine et al. (2015) Bouilly et al. (2015);

	p. Gly35Glu; p. Arg92Gln; p. Cys497Valfs*53; p. Arg44Leu; p. Gly91Trp; p. Gly111Arg; p. Arg117Trp; p. Lys371Thr; p. Pro619Leu				
NR5A1	p. Tyr5Asp; p. Lys372del; p. Leu231_Leu233del; p. Pro237Leu; c. 666delC; c. 390delG; c. 825C > T; c. 225G > C; p. Asp293Asn; p. Met1Ile; p. Leu231_Leu233del; p. Arg255Lys; p. Gly146Ala; p. Pro136Leu; p. Ala192ser; p. Pro195Leu; p. Ala192Val; p. Arg313His	Nuclear receptor subfamily 5, group A, member 1	Steroidogenesis in the ovary	WES	Jiao et al. (2017) (4); Eggers et al. (2015) (2); Lourenco et al. (2009); Philibert et al. (2013); Janse et al. (2012)
NUP107	c.1339G > A	Nucleoporin 107 kDa	mRNA transport and translation	WES	Weinberg-Shukron et al. (2015) (2);
PGRMC	p. His165Arg	Progesterone receptor membrane component 1	Hormone pathway regulation, meiotic recombination of cell cycle gene, and apoptosis regulation	CGES	Mansouri et al. (2008) (2)

(continued)

Table 7.1 (continued)

Genes	Mutation	Full name	Function	Study type	Reference
POLG	p. Tyr955Cys; p. Arg943His	DNA polymerase gamma	DNA replication	GWAS	Pagnamenta et al. (2006); Tong et al. (2010)
POLR3H	c.149A > G	RNA polymerase III subunit A	Cell cycle, growth, and differentiation regulation		Franca et al. (2019) (4)
PSMC3IP	c. 600_602del c. 489C > G; c.268G > C; c.597 + 1G > T; c.206_208delAGA; c.189 G > T	PSMC3 interacting protein	Homologous recombination repair in meiosis	WES	Zangen et al. (2011) (2); Al-Agha et al. (2018); Mei et al. (2022); Sirchia et al. (2022)
SALL4	c.1790A > G; c.541G > A; c.2279C > T; c.2449A > G; c.3149 T > C	Spalt-like transcription factor 4	Oogenesis	WES	Wang et al. (2019) (4); Wang et al. (2009); Jin et al. (2020)
SGO2	c.1453_1454delGA	Shugoshin 2	Transcription factor	WES	Faridi et al. (2017) (4);
SOHLH1	c. 210G > T; c. 530 + 6 T > G; c. 235G > A; c. 314A > G c. *118C > T; c. 950C > T; c. 1126G > A; c.705delT	Spermatogenesis and oogenesis specific basic helix-loop-helix 1	Early folliculogenesis	WES	Qin et al. (2014a, b); Zhao et al. (2015) (3); Jolly et al. (2019) (2)
SPIDR	c.839G > A; c.814C > T;	Scaffolding protein involved in DNA repair	Homologous recombination repair in meiosis	WES	Smirin-Yosef et al. (2017)(2); Heddar et al. (2022)

STAG	c.3052delC; c.659 T > G; c.968delC	Stromal antigen 3	DNA damage, mRNA transport and translation	WES	Heddar et al. (2019) (4); Caburet et al. (2014) (2)
SYCE1	c.613C > T	Synaptonemal complex central element protein 1	Homologous recombination repair in meiosis	WES	De Vries et al. (2014) (4)
TLK1	Intron: chr2: 171053827 (rs149844334) T > C; Intron: chr2: 171160246 (rs113416007) G > A; Intron: chr2: 171056401 (rs17283147) A > T; c. 1158C > T: c. 1217C > T	Tousled like kinase 1 gene	DNA replication	GWAS	Xu et al. (2020)
WT1	c.376C > T; c. 1109G > A; c. 647-27C > T; c. 647-13G > C; c. 647-13G > A; c. 950 + 14 T > C	Wilms tumor suppressor	Granulosa cell differentiation and oocyte-granulosa interaction	CGES	Wang et al. (2015a, b)(2); Gao et al. (2014) (4)

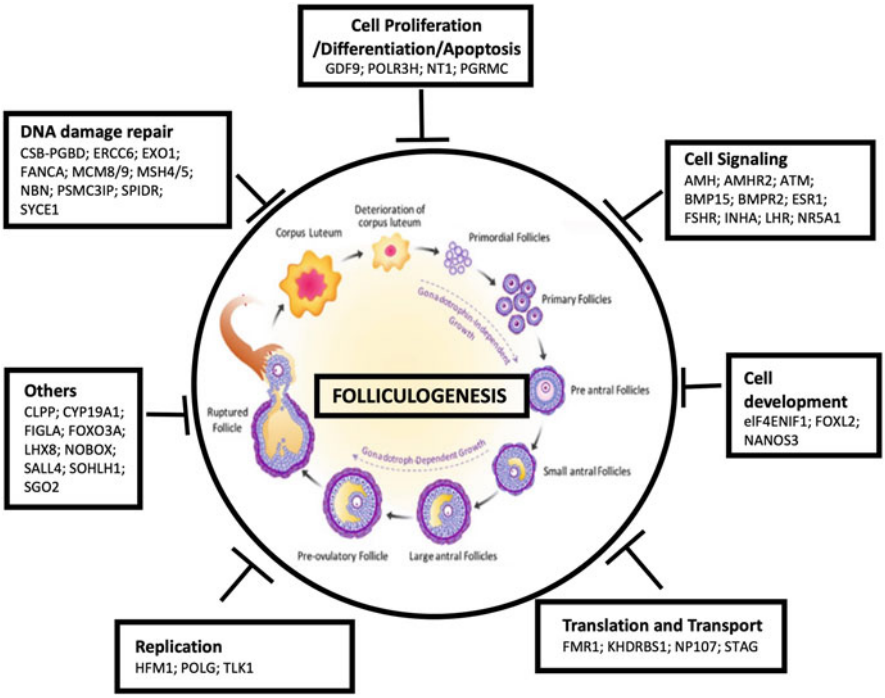


Fig. 7.1 Genes involved in the process of folliculogenesis and ovulation

In the name of genetic testing, most clinics have adopted regular karyotyping in order to detect Turner syndrome, which is common in POF patients. Along with this, the American College of Medical Genetics and Genomics (ACMG-2005) and the American College of Obstetricians and Gynecologists (ACOG-2010) recommended Fragile X premutation analysis in idiopathic POF patients. Apart from these well-known genetic contributors, mutations in a number of other genes have been associated with POF; however, there is no recommendation for their testing in POF. One reason behind this could be the large number of target genes (Nguyen et al. 2017, http://www.iamg.in/genetic_clinics/full_text.php?id=257). In this chapter, we'll talk about the various genetic tests that have helped or are helping to discover the disease's underlying genetic causes.

7.2 Genetic Testing in POF

7.2.1 Karyotyping

Due to the involvement of X-chromosome abnormalities (translocation or deletion) or mosaicism (monosomy or trisomy) in POF, karyotyping and cytogenetic evaluation becomes important tools (Di-Battista et al. 2020; Sala et al. 1997; Sherman

2000; Goswami and Conway 2005; Moysés-Oliveira et al. 2015; Xue-Jiao et al. 2012). X-chromosome structure and numerical effects are seen in about 13% of the POF cases (Shelling et al. 2000; Qin et al. 2014a, b; Cordts et al. 2011). A monosomy condition like Turner syndrome (45X), which is associated with gonadal dysgenesis, short stature, and primary amenorrhea in 1:2500 affected females, results in POF (Hook and Warburton 2014). Various studies have shown that women suffering from Turner syndrome have a loss in oocyte number due to accelerated follicular atresia (Abir et al. 2001; Lunding et al. 2015). Trisomy in X (47 XXX) that hits 1:1000 females, results in hypogonadotropic POF (Cordts et al. 2011).

Another X-chromosome abnormality that causes POF is a premutation condition of the Fragile X-mental Retardation (FMR1) gene located on Xq27.3 (Goswami and Conway 2005). The FMR1 gene plays a vital role in fetal ovary germ cells and in the maturing follicle's granulosa cells (Hergersberg et al. 1995; Allingham-Hawkins et al. 1999; Allen et al. 2007). FMR1 protein functions by binding with RNA to suppress its translation so that it can inhibit the overexpression of genes having a role in oocyte development, which eventually led to a decreased follicular pool (Cordts et al. 2011, Dixit et al. 2010). The 5' UTR region of the FMR1 gene consists of CCG repeats, of which <45 repeats are considered normal, 55–199 are considered premutations, and $>$ 200 are full mutations that result in Fragile X syndrome in males (Dixit et al. 2010). The premutation stage increases the chances of POF in women due to an increase in follicular atresia. The frequency of FMR1 premutation is 6% in sporadic and 13% in familial cases of POF (Wood and Rajkovic 2013).

Cytogenetic analyses suggest that the long arm of the X-chromosome is also critical for ovarian development and function (Powell et al. 1994). There is a common breakpoint in the regions of the long arm due to deletions in Xq24-Xq27 (POF1) and frequent translocations between Xq13 and Xq21 (POF2) (Powell et al. 1994, Eggermann et al. 2005, Davison et al. 2000, Summitt et al. 1978). Fifty percent of the translocations on the X-chromosome result in POF due to gene disruption, meiosis errors, or the position effect (Therman et al. 1990). However, most of the breakpoints in the translocation of Xq21 were gene-free regions, except for two genes (DACH2, POF1B), which have regulatory roles and could contribute to POF (Bione et al. 2004). More studies need to be undertaken to investigate the roles of these genes/regions and their translocations in causing POF.

Some cases of POF may require further in-depth investigations to identify the genetic defects with high accuracy. For example, in some cases of mosaicism in monosomy or trisomy, the diagnosis was done using karyotyping followed by FISH (Sugawara et al. 2013). In a study on two POF patients, X trisomy was detected by G-banding karyotyping and fluorescence in situ hybridization (FISH) with X and Y-bearing probes (Sugawara et al. 2013). Another study on 1000 POF patients performed FISH along with karyotyping for low-level mosaicism in monosomy of the X-chromosome. The karyotyping analysis discovered 108 chromosomal abnormalities; however, in 23 patients with normal 46 XX karyotypes, FISH analysis revealed greater levels of X-monosomy in cells (Lakhal et al. 2010). Usual clinics adopt karyotyping after or along with hormonal profiling, which may be insufficient to diagnose other genetic abnormalities associated with POF.

7.2.2 Candidate Gene Sequencing

Apart from the chromosomal numerical and structural abnormalities, polymorphisms/mutations in genes associated with normal ovarian function have been reported in POF. This includes polymorphisms identified by candidate gene sequencing, such as in the FOXL2 (Shelling et al. 2000; Harris et al. 2002), NR5A1 (Philibert et al. 2013; Lourenço et al. 2009), BMP15 (Dixit et al. 2004; Chand et al. 2006; Di Pasquale et al. 2006; Dixit et al. 2006; Laissue et al. 2006), GDF9 (Kumar et al. 2017), and INHA (Marozzi et al. 2002) genes.

7.2.2.1 Oocyte-Specific Transcription Factors Genes

Oocyte-specific transcription factors are important for primordial follicle development and their differentiation (Qin et al. 2014). Some of the transcription factor genes like NR5A1, NOBOX (newborn ovary homeobox gene), FIGLA, and FOXL2 have been investigated in POF. NR5A1 (steroidogenic factor 1) located on 9q33.3 acts as a nuclear receptor required for gonadal differentiation. It regulates other genes like STAR, CYP11A1, CYP17A1, CYP19A1, LH, and INHA that regulate steroidogenesis (Lourenco et al. 2009, Persani et al. 2010). Mouse model studies on NR5A1 knockout showed a decrease in oocyte number, leading to infertility (Jeyasuria et al. 2004). However, one study on 20 subjects found mutations (p. Asp293Asn; p. Met1Ile; p. Leu231_Leu233del; c. 666delC; c. 390delG) causing severe loss in activation of NR5A1 and conditions like 46, XX, which are common in POF (Lourenco et al. 2009). NOBOX and FIGLA (factor in germline alpha) are both important in the initial process of folliculogenesis (Rajkovic et al. 2004). In fact, NOBOX controls the expression of many genes involved in follicular development. In a mouse model study on NOBOX knockout, folliculogenesis-specific genes such as Oct 4 (octamer-binding transcription factor 4) and GDF-9 (growth differentiation factor 9) were downregulated (Rajkovic et al. 2004). In another mouse NOBOX knockout study, hastened oocyte loss and features similar to idiopathic POF were seen (Persani et al. 2010). A study on 96 Caucasian POF patients identified seven known and four novel single nucleotide polymorphisms, including p. Arg355His and p. Arg360Gln in the NOBOX gene (Qin et al., 2007), but mutations in this homeobox were not found in another study on Japanese and Chinese patients (Zhao et al. 2005, Qin et al. 2009).

The knockout of another transcription factor, factor in germline alpha (FIGLA), which controls numerous oocyte-specific genes and the genes encoding components of the zona pellucida, replicated the phenotype of POF in mice by causing the loss of primordial follicles and oocytes (Bayne et al. 2004; Soyal et al. 2000). Three FIGLA gene polymorphisms (c.11C > A; 15-36del; c.419-421del) were identified to show significant association with POF after investigation on 100 patients (Zhao et al. 2008), suggesting FIGLA to be an important candidate gene for POF.

Forkhead box protein L2 (FOXL2) is expressed in the granulosa cells throughout embryogenesis and adult life (Harris et al. 2002). The FOXL2 gene mutations result in blepharophimosis, ptosis, and epicanthus inversus syndrome type 1 (BPES T-1), which is associated with POF (Woad et al. 2006, Crisponi et al. 2001). The incidence

of FOXL2 mutations in idiopathic POF patients was approximately 5%, irrespective of BPES (Harris et al. 2002). In a study including 70 POF patients from Slovenia and New Zealand, the mutation (772 T > A) was found in the Slovenian patients, while the deletion (661690del) was found in the New Zealand patient (Harris et al. 2002). A missense mutation (1009 T > A) and deletion (898927del) in the FOXL2 gene were discovered in a study of 70 patients with idiopathic POF (Bodega et al. 2004). In an Indian study of 80 POF patients, 773C > G and 738C > T mutations in the FOXL2 gene were discovered that were absent in the control group, demonstrating the importance of this gene in the development of POF (Chatterjee et al. 2007). However, a mouse gene knockout analysis of the FOXL2 gene indicated its role in granulosa cell differentiation and its absence leading to a depletion of primordial follicles, which provided support for these association studies (Schmidt et al. 2004).

Another gene, LIM homeobox gene 8 (LHX-8), encodes a transcriptional regulator important for germ cell development and oogenesis. LHX-8-knockout mice showed disturbed expression of genes such as GDF9 and NOBOX (Qin et al. 2008); however, a study on 95 Caucasian POF patients identified c. 769 + 10G > T and c. 1787A > G polymorphisms that were common with controls in the study (Rossetti et al. 2017). Therefore, further studies are needed to identify functionally relevant polymorphisms in this gene.

Research has underlined the importance of spermatogenesis and oogenesis-specific basic helix-loop-helix (SOHLH 1 and 2) in the disease, as they are expressed from the primordial follicle till the primary follicle stage (Qin et al. 2014b; Dixit et al., 2004; Watkins et al., 2006; Gallardo et al., 2008; Zhao et al., 2015; Patiño et al. 2017). From the knockout study of these two factors, it was found that there was defective primordial cell differentiation, fastened loss of follicles, and the absence of oocytes within 6 weeks of age (Choi et al. 2008). A study on Chinese and Serbian POF patients identified eleven different variants in the SOHLH-2 gene, out of which four were deleterious. Out of these eleven variants, four (c. 210G > T; 530 + 6 T > G; 235G > A; 314A > G) showed an association with ovarian failure (Qin et al. 2014a, b). In another study on 364 Chinese POF patients, there were two missense variants (c. 950C > T; 1126G > A) and one mutation (c.*118C > T) in the 3' UTR of the gene that is thought to affect the functioning of the SOHLH1 gene, contributing to the disease (Zhao et al. 2005; Zhao et al. 2015). However, large population studies from different ethnicities involving these transcription factors have not been undertaken to test their association with POF.

7.2.2.2 Folliculogenesis Growth Factor Genes

Folliculogenesis factors, including BMP-15, GDF-9, and INHA, may affect the development and maturation of follicles. BMP-15 found on Xq11.2 belongs to the transforming growth factor-beta (TGF-beta) family, which is responsible for the growth and differentiation of oocytes (Hashimoto et al. 2005). Folliculogenesis and follicle maturation are initiated by BMP15 due to its control over the differentiation and proliferation of granulosa cells. The role of BMP15 in folliculogenesis is supported by mouse knockout studies (Tiotiu et al. 2010) and haploinsufficiency, which highlighted its role in granulosa cell signaling and antiapoptotic role in

maintaining the follicular pool (Rossetti et al. 2009). A study of 166 idiopathic Caucasian people found p.Y235C, p. R68W, and p.A180T polymorphisms in the BMP15 gene to be associated with POF (Di Pasquale et al. 2006). When 133 POF patients were screened for the mutation in the BMP15 gene, 16 novel variants were identified. A full list of the variants identified is mentioned in Table 7.1 (Dixit et al. 2006). Another study of 52 idiopathic POF patients found c. 510_512delT mutation to be associated with the disease (Kumar et al. 2017). In human studies, BMP15 polymorphisms (788ins TCT; p. 262ins Leu; 5'UTR-31 T > G; 5'UTR-71C > G; exon1-387G > A) were often observed in control individuals as well (Tiotiu et al. 2010; Di Pasquale et al. 2006; Chand et al. 2006), which makes it a less attractive causative factor; nevertheless, one can suggest that these common variants contribute to POF with the help of other predisposing factors (Tiotiu et al. 2010).

GDF-9, located on 5q23.2, helps in the regulation of primordial follicle development, and the maturation and proliferation of granulosa cells (Persani et al. 2011; Chand et al. 2006; Wang et al. 2010a, b). In mouse deletion studies on GDF-9, it was found to play an important role in primordial follicular development (Dong et al. 1996; França et al. 2018). A study on 127 Indian POF patients targeting the GDF9 gene found variants, such as c. 199A > C and c. 646G > A, which were absent in the control group. They also found a variant c. 147C > T that showed a strong association with POF (Dixit et al. 2005). Another study on 203 POF patients in France found a mutation (c. 557C > A; p. S186Y) in the gene that produces a misfolded pro-region protein that results in POF (Laissue et al. 2006). However, it is still not included as the major cause of POF due to limited data in various POF studies (Laissue et al. 2006; Takebayashi et al. 2000; Dixit et al. 2005).

INHA (inhibin alpha), which belongs to the superfamily of transforming growth factor-beta (TGF-beta) genes, is initially produced by the granulosa cells to diminish the production of FSH and inhibit inhibin production that eventually results in increased follicular atresia, depicting its regulatory role in folliculogenesis (Rah et al. 2014, Shelling et al. 2000). The INHA gene polymorphisms 769G > A, 16C > T, and 124A > G have shown significant association with POF (Shelling et al. 2000; Dixit et al. 2004; Marozzi et al. 2002). The two variants 16C > T and 124A > G were found in combination in Korean POF women (Rah et al. 2014). However, it was absent in Italian and German subsets (Corre et al. 2009) and later on was supported by meta-analysis on this gene that too found no association of these variants in causing POF (Chand et al. 2010; Zintzaras 2009).

7.2.2.3 Ovarian Steroidogenesis Genes

Since gametogenesis is strictly regulated by steroid hormones and variation in their levels or functions might result in ovarian dysfunction, a hormonal evaluation is an essential part of POF diagnosis, and patients suffering from POF generally have less FSH. The genetic aspect of this can be mutations in the follicle-stimulating hormone receptor (FSHR) (Woad et al. 2006) or other hormone receptors that are essential for the regulation of follicular development. In a mouse knockout study of FSHR, there was defective folliculogenesis and a lack of puberty in mutant animals (Abel et al.

2000). Although many studies on POF patients found this rare mutation to have a role in causing the disease (Woad et al. 2006, Pu et al. 2014).

A study on six Finnish families identified a 566C > T (exon 7) mutation in the FSHR gene, which, upon further analysis, was found to be restricted to the Finnish population only (Aittomäki et al. 1995, Da Fonte Kohek et al. 1998, Layman et al. 1998). In another study on New Zealand POF patients in which exons 7 and 10 of the FSHR gene were screened for mutations, no common mutations were identified. However, a novel heterozygous mutation in exon 10 (c.1411A > T) was identified in one patient, and the study concluded that variation in these exons is rarely associated with POF (Woad et al. 2013).

Estrogen receptor 1, located on 6q25.1, is a transcription factor that regulates cell proliferation and growth in several organs, including the thecal and granulosa cells in the ovary (Lee et al. 2009, de Mattos et al. 2014). Follicular maturation, growth, and apoptosis are mostly controlled by the estrogen receptor (M'Rabet et al. 2012). In a knockout study, an arrest in follicular biogenesis was reported, leading to a huge number of atretic follicles at 22 days of age (Drummond and Findlay 1999). Common mutations in the ESR1 gene, such as 397 T > C and 351A > G, showed a linkage with an increased risk of POF (Bretherick et al. 2008, Cordts et al. 2012). However, one study on ESR1 gene polymorphisms (397 T > C; 351A > G) showed contradictory outcomes (Silva et al. 2010).

A few other genes that have been investigated in POF but do not appear to affect the POF risk are HELQ, PRIM1, UIMC1, and WRN. Some of these genes are known to affect menopause and participate in DNA damage repair and replication (Stolk et al. 2012). Screening of HELQ in 192 Chinese POF patients found SNPs that were shared with control individuals (Wang et al. 2015a, b). Another study screened PRIM1 in 192 Han-Chinese women with POF but found no mutation in the patients (Wang et al. 2016). Analyses of the UIMC1 gene in 200 Iraqi POF patients identified mutations in the genes, but they did not show an association with the disease (Shaker and Mohammed 2020). Some of the other sequencing studies that targeted candidate genes are mentioned in Table 7.1.

Initially, the target genes for POF were selected based on their functions in normal ovarian function, but since a large number of cases were idiopathic, large-scale polymorphism and sequencing studies promise to identify a greater number of genes affecting the risk of POF.

7.2.3 Genome-Wide Association Studies (GWAS)

Ovarian functions and POF are multigenic processes. Single-gene studies somehow helped in the identification of a few genes, yet they failed to identify novel genes that were not directly related to ovarian functions. GWAS have helped reveal the multigenic nature of POF. Initially, many GWAS studies identified genes associated with menopausal age, and many loci were significantly associated with age during natural menopause. These studies suspected 17 new loci on various chromosomes associated with hormonal balance, immune activity, and DNA repair (He and

Murabito 2014, He et al. 2009, Stolk et al. 2009, Stolk et al. 2012). GWAS studies on POF targeted these genes that regulate natural menopause as they could affect susceptibility to POF. The ReproGen consortium undertook the largest GWAS study to relate POF and menopause with the help of a mouse model that added up fivefold ovarian aging-linked loci, which might help in further phenotypic extremes in POF (Ruth et al. 2021).

Another GWAS study in 101 Korean cases suggested the parathyroid hormone-responsive B1 (PTHB1) gene to be a candidate for POF (Kang et al. 2008). However, the link between this gene and the POF is still unclear, but this can be associated with the mutual Bardet-Biedl syndrome that represents both PTHB1 variants and the POF phenotype. Thus, PTHB1 can also be considered a strong candidate for POF (Mukerjee and Dorfman, 2014; Chapman et al. 2015). In yet another GWAS study, ninety-nine idiopathic Caucasian POF women were screened, which identified the ADAMTS19 gene as another candidate gene for POF. Previous studies on mice showed its association with gonadal differentiation (Knauff et al. 2009). However, its linkage with POF was not confirmed in another investigation involving 60 POF patients, making it a less appealing candidate gene for POF (Stigliani et al. 2013). GWAS studies in POF patients involved relatively small sample sizes, which leaves the genes in question for further investigations.

7.2.4 Next-Generation Sequencing (NGS)

Even though unbiased genome analysis has sped up the finding of novel candidate genes and genetic risk factors through the implementation of GWAS, no genes that can be called very strong candidates for POF have been found (Chapman et al. 2015). Whole exome and genome sequencing are the most widely used forms of NGS for idiopathic cases. WES studies have identified STAG3, HFM1, MCM8, and MCM9 as the candidate genes in POF (Wang et al. 2014; Wood-Trageser et al. 2014; Caburet et al. 2014; AlAsiri et al. 2015). Another exome sequencing study on 12 idiopathic unrelated individuals and 70 POF-causing genes has identified the role of the ADAMTS19 and BMPR2 genes in POF (Fonseca et al. 2015). ADAMST19 has consensus with the findings of GWAS, making it a strong candidate gene for POF. This study has also helped in supporting the two known genes (BMP-15 and GDF-9) as strong candidates due to their downstream involvement in BMPR2 signaling.

Maternal mitochondria and their genes are already believed to be an important part of reproductive aging and infertility. The identification of some key mitochondrial genes, which may play a significant role in the development of POF, has also been made possible by whole exome sequencing. These genes are important as they help in oogenesis by providing energy, calcium homeostasis, and apoptosis (Wai et al. 2010). Mitochondrial genes, such as DNA polymerase gamma catalytic subunit (POLG), leucine-rich pentatricopeptide repeat-containing (LRPPRC), leucyl-tRNA synthetase-2 mitochondrial (LARS2), caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP), alanyl-tRNA synthetase 2 mitochondrial (AARS2), and

twinkle mtDNA helicase (TWNK), were found to show association with POF (Tiosano et al. 2019). Specific polymorphisms in the POLG (c. 2890 C > T), CRPPRC (c. 7G > T; c. 2965C > T), and LARS2 (c. 1565C > A; c. 1077delT; c. 1886C > T) genes were identified by various WES studies to affect disease progression (Pierce et al. 2013; Chen et al. 2018; Liu et al. 2020). With the increase in cost-effectiveness of NGS, it is believed to play a critical role in identifying genetic polymorphisms that affect susceptibility to POF.

7.2.5 Array Comparative Genomic Hybridization (Array CGH)

Another strategy for identifying genomic structural variations is array comparative genomic hybridization, which identifies genomic changes in terms of deletion, duplication, or copy number variations (Shelling and Ferguson 2007). A study on CNVs in sporadic and familial POF patients identified CNVs in five genes, which were involved in reproductive diseases (DNAH5, NAIP, DUSP22, NUPR1) and folliculogenesis (AKT1) (Aboura et al. 2009). In another study, array CGH detected 11 new CNVs in 26 POF patients, of which one was in the GDF9 gene and the remaining ten were in other novel candidate genes (Norling et al. 2014). Another array CGH study on 44 POF patients found 24 rare CNVs and reported 44 regions of deletions and duplications in the genes having roles in meiosis (MAP4K4, PLCB1, and RB1CC1), folliculogenesis (IMMP2L, FER1L6, and MEIG1), and DNA repair (RBBP8) (Ledig et al. 2010). Most of the genes discussed above are autosomal, suggesting a major role for chromosomes other than the X-chromosome in the etiology of POF (Stigliani et al. 2013, Christin-Maitre and Tachdjian 2010). Autosomal genes, SYCE1 and CPEB1, which have roles in DNA-double strand break repair and cell progression during mitosis were suspected to be POF candidate genes as their knockout mice displayed germ cell apoptosis and oocyte loss, leading to follicular deficiency. This was confirmed by a study on 89 POF patients with microdeletions in these genes (McGuire et al. 2011). However, most of the above studies lack consensus regarding the CNVs that are the best candidates for POF.

7.2.6 Microarray Analysis

Small RNAs, like other human diseases, get dysregulated in POF patients as well. Several miRNAs have been identified to play roles in ovarian development and folliculogenesis (Ahn et al. 2010, Veiga-Lopez et al. 2013). A microarray miRNA study on three POF patients in comparison with controls identified an elevated mir-23a level, which was predicted to have a role in granular cell apoptosis (Yang et al. 2012). Unfortunately, its significance remained unclear due to the small sample size used in this study. In another microarray study on 140 Chinese POF patients, 22 upregulated and 29 downregulated mi-RNAs were identified (Dang et al., 2015). Among them, miR-22-3p, which had an inverse relationship with FSH, was thought to significantly contribute to POF (Dang et al. 2015). Similar to the above, other

Table 7.2 miRNAs associated with POF

miRNA	Association with POI	Methods used	Reference
miR-23a	Apoptosis in granulosa cell	Microarray	Yang et al. (2012)
miR-22-3p	Decrease in ovarian reserve	Microarray	Dang et al. (2015)
miR-379-5p	Granulosa cell development	Microarray	Dang et al. (2018)
miR-127-5p	Upregulated miR-127-5p in POI patients	Microarray	Zhang et al. (2020)
miR-196a2	Interfere in mRNA expression of granulosa cell	Non-microarray	Rah et al. (2013)
miR-146	Upregulated miR-146 and interfere with mRNA expression of granulosa cell	Non-microarray	Rah et al. (2013); Chen et al. (2015)
miR-21	Downregulated miR-21 in POI patients	Non-microarray	Li et al. (2020)

studies identified alterations in another miRNA in POF, which are detailed in Table 7.2. Another microarray analysis on 30 POF patients found downregulation of HCP5, a long noncoding RNA (lncRNA), which accelerated apoptosis and damaged the DNA repair system. HCP5 is co-localized to MSH5, a POF-causing gene, and its downregulation results in lower expression of MSH5 (Wang et al. 2020). However, there were many other mi-RNAs that were identified by non-microarray analyses, such as qPCR or luciferase assay, in POF patients (Rah et al. 2013; Chen et al. 2015; Li et al. 2020). Thus, noncoding RNAs may be used as biomarkers of POF, but further large-scale studies are required.

7.3 Conclusions and Future Perspective

Only a few candidate genes for POF have been identified. The reason behind the unidentified candidate genes for POF might be a major percentage of idiopathic patients. Usual tests in clinics for POF include amenorrhea, family history, sonography, hormone (FSH) analysis, and karyotyping. Since chromosomal abnormalities are seen in about 10–13% of POF patients, the most widely used test is karyotyping, which helps in detecting chromosomal mosaicisms. However, since karyotyping could identify the cause in only a few patients, other diagnostic techniques such as candidate gene sequencing, GWAS, array CGH, microarray, and next-generation sequencing are employed for the genetic diagnosis of the disease. While candidate gene studies have identified polymorphisms in several genes, there are only a few genes that were found to be common across different studies, which include the ADAMTS19, BMP-15, and GDF-9 genes. Functional validation in a large number of patients would be required to get a clear picture. There is a lot to be done in the field of diagnosis of POF to reach a particular gene or a set of genes as well as

to bring it into practice in clinics. Since single-gene studies are largely restricted to the identification of a few genes, large-scale sequencing, microarray, exome sequencing, or even whole-genome sequencing would need to take a deeper dive to uncover other genetic causes of POF.

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Microbiome Testing in Female Infertility

8

Anshita Sharma and Rajender Singh

Abstract

Residential commensal microorganisms in the female reproductive tract act as an interface between the host and the outer environment to manifest beneficial effects. Alterations in microbiota diversity and abundance cause dysbiosis, resulting in microflora disequilibrium, which is linked to the emergence and progression of many diseases, including abnormal vaginal discharge, endometritis, salpingitis, parametritis, oophoritis, tubo-ovarian abscess, pelvic peritonitis, and perihepatitis. The most common recurrent vulvovaginal infections (RVVI) include trichomoniasis, vulvovaginal candidiasis, bacterial vaginosis (BV), and other dysbiosis-linked diseases. Female reproductive tract dysbiosis may result in infertility, miscarriage, or preterm delivery. Culture-based methods dominated the field of microbial dysbiosis testing for a long time. Because of the limitations of culture-dependent microflora identification, modern tools such as PCR, microbiome sequencing, and metabolomics have replaced classical methods of dysbiosis detection. This chapter highlights the impact of female genital tract dysbiosis on fertility and gynecological diseases and the contemporary methods of diagnosis along with an emphasis on next generation sequencing-based diagnostic technology.

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Dysbiosis · Microbiome · *Lactobacillus* · Metagenomics · Female reproductive tract dysbiosis · Genetic testing · Reproductive medicine · Infertility

8.1 Introduction

The consortium of microorganisms inhabiting the human system inside or on the surface in a mutually associated manner makes humans fundamentally known as superorganisms (Salvucci 2019). The vast majority of commensal and symbiotic microorganisms comprising bacteria, actinomycetes, archaebacteria, and fungi, together harboring the human niche, are collectively known as the microbiota, and the ecosystem they thrive in is the microbiome. The microorganisms in commensalism with the human female genital tract ambience are referred to as the female genital microbiota, manifesting in the upper and lower reproductive parts to benefit the machinery via maintaining homeostasis, protecting against overt pathogenic invasion, and safeguarding from pH and temperature alterations. Studies on the microbiome, specifically under the Human Microbiome Project (HMP), launched in 2005 in the USA by the National Institutes of Health (NIH), have led to a comprehensive understanding of the therapeutic strategies revolving around the microbiome in human disease prevention (Grice and Segre 2012). It provides precise information on the community composition, dynamicity, and genome sequence of microorganisms present in the gut, genital tracts, and oral cavity.

In this book chapter, we will learn about the human female genital tract microbiome, its association with infertility, associated severe pathologies, and currently available diagnostic tools and genetic testing methods.

8.2 Female Genital Tract Microbiota

The human female genital tract, covering about 9% of the total human microbiota, creates a haven for diversity of microbes by acting as an interface between the host and the genital tract, which varies in composition depending upon the age, BMI, lifestyle, and hormonal changes. The composition and abundance of microflora are ever-changing throughout a woman's life during puberty, menstruation, gamete formation, conception, pregnancy onset, and gestation and are responsible for microbiome establishment in the next generation. The vagina confers residence to 10,000 times more bacteria than those present in the upper reproductive tract, including the uterus and fallopian tubes (Chen et al. 2017). The vaginal microbiota, which usually progresses from the vagina and endocervix to the endometrium, is dominated by aerobic, facultative anaerobic, and obligate anaerobic species of bacteria. A wide variety of bacteria reside in the upper parts of the tract, mostly growing in mildly alkaline conditions compared to the low pH environment of the vagina, which harbors mainly *Lactobacillus*. *Lactobacilli*, being the most

represented genus, depicts the health of the vagina (Franasiak and Scott 2015; Tamrakar et al. 2007) and is known to shield against pathogenic invasion and infection by lowering the pH in the vaginal tract (Mitchell et al. 2015; Witkin et al. 2007). The metagenomic studies on vaginal flora confirm the predominance of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, and *Lactobacillus jensenii* in a healthy female vagina (Chee et al. 2020). At the phyla level, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are predominant in the endometrium; other observed genera are *Prevotella*, *Bifidobacterium*, *Gardnerella*, *Atopobium*, *Megasphaera*, *Sneathia*, and *Anaerococcus*.

8.3 Dysbiosis: Milieu in Gynecological Diseases and Infertility

Alterations in microbiota diversity and abundance cause dysbiosis, an imbalanced microflora resulting in disequilibrium, which is linked with the emergence and progression of many diseases, trailed by abnormal vaginal discharge, leading to endometritis, salpingitis, parametritis, oophoritis, tubo-ovarian abscess, pelvic peritonitis, and perihepatitis (Fitz-Hugh-Curtis syndrome). This suggests that a healthy microbiota plays a protective role against urogenital diseases, such as bacterial vaginosis, yeast infections, sexually transmitted infections, urinary tract infections (Martin Jr. et al. 1999; Pybus and Onderdonk 1999; Sobel 1999), HIV infection (Ralph et al. 1999), and preterm birth (Nejad and Shafaie 2008), which directly correspond to the malfunctioning of the reproductive system and fertility. The most common recurrent vulvovaginal infections (RVVI) include trichomoniasis, vulvovaginal candidiasis, bacterial vaginosis (BV) and other dysbiosis-linked diseases.

The endometrial microbiota is essential to study as the implantation of embryo takes place in the uterus. An increase in *Enterococcus*, *Fusobacterium*, *Prevotella*, *Actinomyces*, *Gardnerella*, *Propionibacterium*, *Corynebacterium*, *Staphylococcus*, and *Streptococcus* has been associated with endometriosis (D'Alterio et al. 2021). *Olsenella, Dialister, Prevotella, Megasphaera, Lactobacillus,* and *Atopobium* are linked with the occurrence of preterm birth. Increasing disturbance of the lactobacillary flora (lactobacillary grades I, IIa, IIb, and III) is highly correlated with the presence of *Gardnerella vaginalis*, *Trichomonas vaginalis*, enterococci, group B streptococci, and *Escherichia coli. Gardnerella vaginalis* is known to be detrimental to fertility status, resulting in irregular uterine bleeding and histological endometritis. Women with a normal vaginal microbiome are found to be at 75% lower risk of preterm birth than those with the absence of *Lactobacilli*.

BV emerges as a consequence of vaginal lactobacilli being replaced by an overgrowth of *Gardnerella vaginalis*, anaerobes, mycoplasmas, and other typical BV bacteria, such as *Atopobium vaginae* and *Mycoplasma hominis* (Swidsinski et al. 2005; Tamrakar et al. 2007) affecting approximately 29% of women of reproductive age in the USA, with variations prevalent in different races and ethnicities (Allsworth and Peipert 2007). BV conditions the formation of polyamines and other compounds that trigger the release of pro-inflammatory cytokines, interleukin

(IL)-1 β and IL-8 (15), while supporting the overgrowth of anaerobes, which is linked with pelvic inflammatory disease (PID). It is associated with tubal factor infertility (Sweet 1995) and idiopathic fertility, which is connected with inefficacy in sperm viability (Moreno and Simon 2018). *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are the notorious names behind PID (Darville 2021).

Apart from affecting sperm survival and fertility, dysbiosis also affects implantation and sustenance of pregnancy, resulting in miscarriages or preterm birth (Donders et al. 2009). Strains belonging to the phylum Proteobacteria are abundant in the third trimester of pregnancy and are responsible for inflammation (Koren et al. 2012). The abundance of the phylum Verrucomicrobia is prominent in infertility (Komiya et al. 2020). *U. urealyticum* is associated with the production of reactive oxygen species (Potts et al. 2000). *M. genitalium* could be transmitted through sperm to the vaginal environment as it attaches to human spermatozoa. ROS hampers lipid peroxidation mechanisms, membrane integrity and flexibility, adversely affecting sperm tail motion. It results in sperm dysfunction and unsuccessful fertilization, as demonstrated by high rates of miscarriages (Mastromarino et al. 2014).

8.4 Gut Microbiome in Reproductive Health and Fertility

Similar to the vaginal and uterine microbiomes, the gut microbiome also plays a crucial role in reproduction. The gut microbiome regulates the host's fatty acid β-oxidation, which is required for oocyte maturation and embryonic development (Dunning et al. 2010). The maternal microbiome produces short-chain fatty acids (SCFAs) that pass the placenta and aid in differentiation signaling of neural, intestinal, and pancreatic cells through embryonic GPR41 and GPR43 (Kimura et al. 2020). Gut metabolites produced by such bacteria bring disruptive modifications to innate immunity, such as butyric- and propionic acid-producing bacteria that ameliorate NF-kB transactivation and IL-8 secretion. A significant role of the gut microbiome in sustaining pregnancy and hormonal communication is well established (Aagaard et al. 2012).

An excess of gram-negative bacteria in the gut elevates the chances of inflammation, increasing the risk of infertility (Barrett and Taylor 2005). Gut microbiome manifestations in obesity and inflammatory diseases result in infertility directly or indirectly by impairing oocyte maturation and altering spindle morphology or via PCOS. *Bacteroides vulgatus* is known to rise in the gut microbiota of PCOS patients, corresponding to a decrease in the levels of glycodeoxycholic acid and tauroursodeoxycholic acid (Qi et al. 2019). *B. vulgatus* plays an important role in modulating the conversion pattern of bile acids and decreasing ILC3-produced IL-22, which has its own therapeutic significance in the estrous cycle, ovarian dysfunction, and insulin resistance. MTHFR mutations and vitamin B deficiency are instrumental in leading to homocysteine imbalance and causing infertility by impairing implantation and fetal viability (Altmäe et al. 2010). The only natural source of vitamin B12 in the human body is its synthesis by microorganisms in the gut.

8.5 Classical Diagnostic Methods

The common diagnostic methods of identifying dysbiosis are (i) the Amsel criteria, which is a set of four parameters including (a) vaginal discharge, (b) whiff test checking for fishy odor with or without the addition of 10% KOH, (c) elevated pH >4.5, and (d) detecting the presence of adherent bacteria on epithelial cell microscopy, with 3 of 4 criteria required to make a positive diagnosis (8), or (ii) gram stain Nugent scoring and modified spiegel criteria (MSC), which relies on microscopy after gram staining of a vaginal smear. The Nugent score is typically based on the presence of large gram-positive bacilli (*Lactobacillus* morphotypes), small gram-variable bacilli (*G. vaginalis* morphotypes), and curved gram-variable bacilli (*Mobiluncus* spp. morphotypes) (Fig. 8.1). The scoring system as Nugent score 0–3 is an indicator of the normal vaginal microbiota, 4–6 indicates intermediate-grade bacterial colonization, and 7–10 is indicative of BV/VD. Undemanding methods involve checking the quality of vaginal swabs, vaginal pH, and wet mount of vaginal fluid, as they form the most basic criteria of diagnosis for *Lactobacillary* grade. Vaginal pH, which is claimed to be 4–4.5 or less, denotes the absence of vaginitis, whereas a pH value of more than 4.5 denotes vaginitis and bacterial vaginosis. Vaginal pH is measured using the VpH glove and is a solid diagnostic tool in daily practices (Lin et al. 2021). Nugent score and qPCR are used

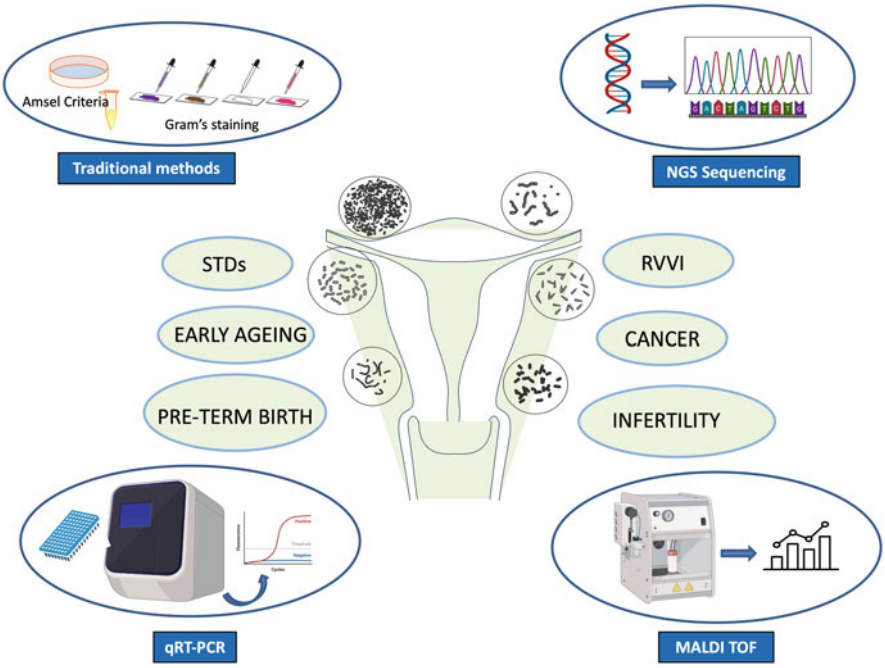


Fig. 8.1 Figure showing the consequences of altered microbiota and techniques currently employed to detect dysbiosis

Table 8.1 Classification of bacteria as CST for determination of microflora

Sr. no.	CST type	pH	Characterizing abundant bacteria	SAI value	Remarks
1.	CST-I	*<4.0*	*Lactobacillus crispatus*	0.558	Very stable, changes in menses, pregnancy only
2.	CST-II	*4.4*	*L. gasseri*	0.242	Dynamic, changes to CST-I
3.	CST-III	*>4.5*	*L. iners*	0.451	*Pro-inflammatory markers*
CST-IV		*>5*	Facultative anaerobes, debatable, linked with BV	0.333	Most dysbiotic state; high dispersion, risk of BV
4.	CST IV-A		*Anaerococcus, Peptoniphilus, Corynebacterium, Prevotella, Finegoldia* and *Streptococcus*		Lower Nugent score
5.	CST IV-B		*Atopobium, Gardnerella, Sneathia, Mobiluncus, Megasphaera*		Higher Nugent score (>7)
6.	CST-V	*>5*	*L. jensenii*	0.009	Healthy state

together to assess the vaginal microbiota and pathobionts. Since these techniques rely on culture, they might not be able to isolate or detect fastidious bacteria.

Uncultivated and fastidious bacteria can be detected using culture-independent techniques. It has led to the establishment of unique microbial community state types (CSTs) on the basis of temporal dynamics that can be useful in microbiome typing. CSTs have been classified on the basis of the abundance and composition of vaginal bacterial species in reproductive age, CST-I, CST-II, CST-III, CST-IV, and CST-V as shown in Table 8.1. The concept of CST involves the qualitative investigation of vaginal microflora and tells about BV. Specificity aggregation index (SAI) values are linked with CSTs to depict the composition and diversity quantitatively. The changes in the values indicate transitions in the community from normal and can illustrate the onset of BV.

8.6 PCR Techniques: Targeting Dysbiosis

Speculations based on a species-specific real-time qPCR approach focusing on the dominant vaginal bacteria *Lactobacilli* suggest that there is a need for clarification on the normal host microbiome and its altering patterns in response to variable microbes. Till date, the diagnostic testing protocols are usually confined to microscopic evaluation or real-time PCR that can detect slow-growing, difficult-to-cultivate, or uncultivable microorganisms (Ralph et al. 1999) (Fig. 8.1). The V1–V3 region is unsuitable for *Gardnerella* identification, and the V4 primer pair is widely

used in identifying vaginal microbe consortia (Subramaniam et al. 2016). Quantitative real-time polymerase chain reaction (qRT-PCR) assays for the most prevalent vaginal *Lactobacillus* species and bacterial vaginosis species, *G. vaginalis* and *A. vaginae,* exist. Researchers have introduced novel and accurate phylogenetically branch-inclusive primers and sequencing with broad-spectrum primers that ignore amplification of *Lactobacillus* using LB-blocking oligomers (Lambert et al. 2013). It has been shown that the quantification of specific bacteria by qPCR contributes to a better description of the non-BV vaginal microbiome, but it has also been seen that the PCR information regarding microbiome variation over time is still very limited. BactQuant is a quantitative real-time PCR-based method with 100% sensitivity to qualitatively as well as quantitatively analyze the bacterial load in silico for taxonomical identification and wide coverage. Here, the total bacterial load is measured by taking the total number of 16S rRNA gene copies per swab (Liu et al. 2012). Table 8.2 shows the gene markers commonly used in the identification of specific genera in the vaginal and endometrial microbiomes.

8.7 Next-Generation Sequencing-Based Diagnosis

Due to the limitations posed by the conventional culture-dependent Sanger sequencing methods using the bacterial 16S rRNA gene or fungal ITS (internal transcribed spacer), it has been claimed that the amplification of short-read lengths across the nine (V1-V9) phylogenetically considerable hypervariable regions of the 16S rRNA gene is efficient for the identification and taxonomical classification of different species or genera of both culturable and unculturable bacteria (Z. Liu et al. 2007). These interspersed regions (V1, V2, V3V4, V4, and V5V6) in the 16S rRNA gene are powerful gene identification tools for accurate determination of the interrelationship of bacteria and to construct a phylogenetic tree that unveils the trajectory of how such changes are functional in severe pathologies (Yarza et al. 2014). The hypervariable region V3, which is a 65-nucleotide long sequence from nucleotides 433-497, comprises the maximum number of SNPs between most bacterial species and helps in distinguishing between closely related species. The detection based on V1–V2 is found to be more successful than V3–V4 in the case of *Lactobacillus* species (Kyono et al. 2018), whereas V3–V5 detects *Gardnerella* and *Bifidobacterium* well. V6 holds considerable nucleotide sequence diversity in its sequences and thus, can be targeted for identifying species.

The complex microbial diversity and biological interdependencies can be accurately interrogated with the help of whole-metagenome sequencing and 16S rRNA sequencing (Schriefer et al. 2018). Though it is widely accepted because of its sensitivity, low cost, and high sample throughput, the major limitation lies in primer selection, which can hamper the identification of isolates at the species level. The focus on V3–V4 sequences has led to primer bias; thus, it is suggested to target multiple variable regions of a given taxon's 16S rRNA to maximize the resolution for taxonomic classification. This method is called MVRSION (Multiple 16S Variable Region Species-Level Identification). It is a high-throughput sequencing

Table 8.2 Genes widely targeted for homology measurement along with techniques used for bacterial identification

Sr. no.	Name of the gene	Technique used for identification	DNA sequence similarity	Product of gene	Bacterial taxonomy
1.	rpoB gene	PCR-RFLP	*86%*	RNA polymerase beta-subunit gene	Enterobacteriaceae, (Mollet et al. 1997), *Staphylococcus* spp. (Drancourt and Raoult 2002), *Streptococcus* spp., aerobic gram-positive species, (Drancourt et al. 2004) *Mycobacterium* spp. (Ben Salah et al. 2008), *Corynebacterium* spp. (Khamis et al. 2005)
2.	sodA gene	PCR	78%	Superoxide dismutase	*Streptococci* (Romero et al. 2011), nonenterococcal, *Coxiella burnetii* (Stein and Raoult 1992)
3.	16S rRNA gene	PCR- terminal restriction fragment polymorphisms (T-RFLP)	97%	16S ribosomal RNA	*Bacillus* (Abdo et al. 2006; Janda and Abbott 2007), *Enterococcus* (Patel et al. 1998), *Lactobacillus* (Piotrowska et al. 2016), *Staphylococcus* (Yugueros et al. 2000)
4.	Gap gene	Multiplex PCR	26–97%	Glyceraldehyde-3-phosphate dehydrogenase	*Staphylococcus* sp. (Ghebremedhin et al. 2008)
5.	Tuf gene	PCR	82–99%	Elongation factor EF-Tu	Coagulase-negative *Staphylococcus* (Hwang et al. 2011)

technique for optimizing taxonomical assignment. Shotgun metagenomics has several advantages as it determines the functional relationships between the host and bacteria at species and strain level (Jovel et al. 2016; Walsh et al. 2018). However, there are certain limitations, such as primer biassing for targeting a single hypervariable region and the termination of PCR after the exponential phase. Next-generation sequencing has several advantages, such as parallel sequencing and the generation of large amounts of sequence data, with maximal yields of ~35 Mbp (454 GS Junior), ~2 Gbp (Ion Torrent PGM), ~10-15 Gbp (Ion Torrent Proton), ~10 Gbp (Illumina MiSeq), and ~100 Gbp (Illumina Next Seq 500).

A major disadvantage of NGS-based molecular identification is its high cost, tough adoption at routine levels, lack of availability for the total population, and requirement of skilled manpower. 16S rRNA sequencing might produce unreliable

results when the species are known to have diverged recently. However, with the arrival of circular consensus sequencing (CCS) technology, distinctions between legitimate and artifactual variations are possible, mitigating the limitations. Other molecular techniques, such as quantitative PCR and FISH methods, can be applied to evaluate bacterial coverage (Fredricks et al. 2005).

8.8 High-Throughput Metabolomics

Dysbiosis can be studied by targeting microbial metabolic status and comparing indigenous keystone species in healthy and dysbiotic patients to investigate the stages of dysbiosis (Dietert 2021). Implicit in the emerging techniques, metabolomics has significantly emerged as an effective tool for determining metabolic variation in disease and providing insights on biomarkers at the molecular level. GC-MS for nonpolar compounds and LC-MS for polar compounds in the vaginal niche are performed to compare the relative concentrations of metabolites in vaginal swabs, vaginal discharge, and cervicovaginal lavage (Watson and Reid 2018). With the growing advent of matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), routine microbial and toxin identification has become easier due to its rapid nature (Seng et al. 2013). It has made the identification of the lineage of strains obvious and accessible. MALDI-TOF MS, although developed for the last few decades, was originally used only anecdotally for rpoB and other genes helpful in bacterial identification.

8.9 Conclusions and Future Perspective

The recognition of vaginal and endometrial dysbiosis as new causes of infertility provides an opportunity to detect the microbial factor behind infertility, suggesting the importance of restoring uterine microbiota to favor personalized medicine. As the current diagnostic approach depends on histological examination, microbial culture might produce contradictory results; hence, assessing the changes in the microbiota through various molecular and sequencing techniques may make the diagnosis easier in female reproductive tract dysbiosis. Functional communication between the host and gut microbes is necessary to decode the precise role of the microbiota in infertility.

While multiplex PCR and in-droplet PCR might serve the purpose of bacterial identification and detection more precisely, simultaneous detection of a number of bacterial and fungal species demands a high-throughput technique. Noteworthy, NGS has become the most reliable and easiest way to investigate the composition and variations in microbiome. A futuristic diagnostic method has to rely on multiomics approaches, such as NGS, for delivering to the need for accurate genetic diagnosis of microbial dysbiosis. Concretely, more advancement on the technology front is needed to make diagnosis more precise and sensitive.

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Preimplantation Genetic Testing

9

Sarabpreet Singh and Fiona Olvitta D'Souza

Abstract

The two most crucial factors limiting the success of assisted reproduction are the quality of the embryo and the endometrium. The lacuna in the present morphology-based embryo selection techniques has led to the pursuit of alternative means of identifying healthy embryos and aid their selection for transfer. Preimplantation genetic testing (PGT) is a technique used in assisted reproduction technology (ART) to assess the genetic composition of embryos before transfer. PGT has been instrumental in improving pregnancy rates, live birth rates, and reducing time to pregnancy in couples at high risk of chromosomal abnormalities and genetic disorders. PGT has aided the implementation of single embryo transfer (sET) programs as a selection tool to identify the best embryo for transfer and has led to significant improvements in fertility treatments worldwide. This chapter details various sampling techniques, indications, and the evolution of analytical techniques that made PGT available to infertile patients. Applications of PGT are now expanding to include polygenic diseases and de novo conditions. This chapter aims at providing an update on preimplantation genetic testing.

Keywords

Assisted reproduction technology (ART) · Embryo biopsy · Recurrent implantation failure · Preimplantation genetic testing (PGT) · Genetic diseases · Congenital disorders · Reproductive Genetics

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9.1 Introduction

Assisted reproductive technology (ART) has witnessed unprecedented advancements in technology in the past four decades, with the ultimate goal of increasing the chances of a healthy conception. Embryo quality assessment has always played a pivotal role in determining the outcome of ART cycles. Limitations of morphological characterization in depicting the genomic composition of preimplantation embryos, along with the limited success rate of ART, led to genetic testing in embryos becoming an integral part of ART.

Preimplantation genetic testing (PGT) offers couples an opportunity to ensure the euploid composition of their embryos prior to transfer, thereby increasing the chances of a healthy pregnancy. Since its advent in 1990 (Handyside et al. 1990), genetic testing in preimplantation embryos has paved the way for significant achievements in delivering healthy babies from parents who are carriers of genetic disorders. Integration of PGT into an ART cycle requires coordination between a team of experts at the ART center, the genetic laboratory, an IVF counsellor, and most importantly, a genetic counsellor to help the patient come to terms with the risks and benefits involved.

The terminology describing genetic testing in embryos was revised by Zegers-Hochschild et al. (2017), from preimplantation genetic screening (PGS) and preimplantation genetic diagnosis (PGD) to the more descriptive term "preimplantation genetic testing" (PGT) (Zegers-Hochschild et al. 2017). PGT is defined as a screening test performed to examine the DNA from mature oocytes (polar bodies) or embryos (cleavage or blastocyst stage) in order to diagnose genetic abnormalities prior to embryo transfer. Depending on the indication and the genetic status of the couple, PGT is now classified into PGT for aneuploidies (PGT-A), PGT for structural rearrangements (PGT-SR), and PGT for monogenic disorders (PGT-M).

9.2 Indications for PGT

A detailed record of the reproductive and family history of a patient is essential to determine their eligibility for PGT as well as in deciding on the most appropriate genetic test to be conducted. Routine genetic screening of all patients opting for ART is not a feasible option given the costs involved. Therefore, a couple choosing PGT in any form must undergo genetic counselling by a professional and certified genetic counsellor to ensure appropriate genetic tests are performed as part of patient screening. This will also determine the type of PGT that will be suitable for the couple.

Advanced maternal age has been significantly associated with an increase in oocyte aneuploidy. In patients under 35 years old, the prevalence of aneuploidy rises to 30–50%; in women over 42 years old, it rises to 80% (Franasiak et al. 2014). Patients facing three or more failed ART cycles despite the presence of good endometrium and morphologically good quality embryos are categorized as recurrent implantation failure (RIF). Patients who have experienced pregnancy loss often

Table 9.1 Indications for PGT

Type of PGT	Indications
PGT-A	Advanced maternal age (AMA) Recurrent implantation failure (RIF) Recurrent pregnancy loss Previous pregnancy with chromosomal disorders Severe male factor infertility
PGT-SR	Inversions Balanced translocations Robertsonian translocation
PGT-M	Carriers of single-gene disorders

undergo genetic testing in the product of conception. Established cases where a whole chromosomal or segmental aneuploidy has been implicated in the previous pregnancy loss are eligible candidates for PGT-A. It is advisable for ART patients experiencing pregnancy loss to undergo genetic testing to determine the etiology of pregnancy loss. Male infertility, such as teratozoospermia and severe oligozoospermia, has been associated with an increased incidence of aneuploidies in sperm, thereby increasing the risk of aneuploidy in embryos (Rubio et al. 2014; Mostafa Nayel et al. 2021). Patients with a history of previous pregnancies with aneuploidies have also been reported to have a higher incidence of embryo aneuploidy and hence can benefit from PGT-A. Indications for PGT are summarized in Table 9.1.

9.3 Embryo Sampling Techniques

In order to deduce the genetic status of the embryo, most PGT methods used to be dependent upon the sampling of genetic material from the oocytes/zygotes (via removal of polar bodies) or embryos (involving biopsy of blastomeres or cells of the trophectoderm). The embryo biopsy is perhaps the most crucial procedure in PGT.

9.3.1 Polar Body Biopsy

A polar body biopsy is usually performed 2–3 hours after oocyte retrieval (<6 h). A biopsy of the polar body is a representative of only the maternal genotype, and aneuploidies of only meiotic origin can be detected. A biopsy of the second polar body may be performed 6–14 hours after fertilization. Alternately, both polar bodies may be biopsied simultaneously on day 1, allowing assessment of both the maternally and paternally contributed aneuploidies. This technique, however, is greatly limited in describing postzygotic mitotic aneuploidies.

9.3.2 Cleavage Stage or Blastomere Biopsy

A cleavage stage biopsy is performed on day 3 embryos (68–72 hours after insemination) at the 6–8 cell stage. A laser-assisted zona opening is followed by the aspiration of a single blastomere. Care must be taken to avoid lysis of the blastomere, as they have much less tensile strength when compared to trophectoderm cells. It is not advisable to perform a biopsy on 4-cell embryos, as this can have a negative impact on embryo development and the inner cell mass (ICM) and trophectoderm (TE) ratio. The use of Ca^{2+}- and Mg^{2+}-free culture medium facilitates blastomere biopsy as it helps reduce cell-to-cell adhesion. A biopsy should not be performed on fragmented embryos (>35%) or embryos with a low development rate.

Though a day 3 biopsy provides time for aneuploidy screening and facilitates fresh embryo transfers, it has several limitations:

1. The removal of too many blastomeres can be detrimental to the development of the embryos as it is known to impede ICM development and can result in a small ICM (Hardy et al. 1993).
2. Aneuploidy results are not very reliable when day 3 embryos are assessed. Day 3 embryos have a significantly higher incidence of aneuploidy and mosaicism when compared to day 5 embryos. In day 3 preimplantation embryos, up to 70% of mosaicism has been reported (Voullaire et al. 2000; Wells and Delhanty 2000; Bielanska et al. 2002).
3. Additionally, the financial burden on the patient also significantly increases as a larger number of embryos will be available on day 3 for biopsy, but not all of them will develop to the blastocyst stage, making their testing redundant.
4. Limitations in polar body and blastomere biopsy have led to large-scale acceptance of trophectoderm biopsy as the standard acceptable procedure for PGT.

9.3.3 Blastocyst/Trophectoderm Biopsy

The blastocyst stage is characterized by the presence of two distinct cell types. The inner cell mass (ICM) gives rise to the fetus, and the trophectoderm (TE) develops into the extraembryonic tissues, the placenta. Trophectoderm biopsies may be performed on day 5 or day 6, depending on the grade of TE expansion. Laboratories performing TE biopsies need to have achieved a high blastocyst development rate and excellent vitrification procedures to ensure an efficient freeze-thaw survival of biopsied blastocysts. TE biopsies are preferred over other invasive sampling procedures for the following reasons:

1. Since the TE cells give rise only to the extraembryonic tissues, the inner cell mass remains untouched.
2. TE biopsies allow the aspiration of 6–8 cells and are a more reliable source of embryonic DNA. DNA yield from TE biopsies allows the diagnosis of single-gene mutations and aneuploidies.

3. Day 5 embryos can withstand biopsy procedures better than early embryos.
4. The survival rate of day 5 embryos post-vitrification is higher than that of day 3 embryos. This also allows a longer time frame to perform genetic diagnostics.

9.4 Factors Influencing PGT Results

Proficiency: TE biopsy is an invasive procedure; however, studies confirmed that when performed by experienced personnel, the procedure does not have a detrimental impact on embryonic viability or implantation potential and does not increase the risk of developing congenital abnormalities and adverse perinatal outcomes (Scott Jr et al. 2013; Li et al. 2022).

The grade of the blastocyst: The preference of embryo selection for biopsy is based on the grade of expansion of the blastocyst and the grade and quality of the TE and ICM. Only embryos considered to have implantation potential based on morphological criteria are selected for biopsy.

Use of laser: TE biopsy procedures include the use of a contactless laser to breach the zona pellucida. Laser-assisted zona drilling may be performed either on day 3 or early day 5 to facilitate herniation of the TE cells or may be performed simultaneously in fully expanded blastocysts during the biopsy procedure. Though the use of noncontact lasers has been deemed safe by numerous studies, it is recommended to use as few laser shots as possible to avoid damage to the genetic material in the biopsied cells.

Technique: The TE cells can be extracted either with laser-assisted slicing, stretching, and flick method or by a combination of these methods. Precaution must be exercised during TE biopsy to avoid aspiration of extruded or fragmented cells, as this may cause misdiagnosis. Aspiration of TE is always performed at 90° (or 180°) opposite to the ICM. In case a re-biopsy is required, it is advisable to wait for re-expansion of the blastocyst to allow clear visualization of the ICM and the previous point of entry.

Repeated freeze-thaw cycles: Multiple freeze-thaw cycles have been known to compromise the implantation potential of blastocysts as well as the outcome of the PGT-A cycle. Blastocysts biopsied and vitrified-warmed once have enhanced clinical outcomes compared to blastocysts biopsied and vitrified-warmed twice (Bradley et al. 2017).

IVF- and ICSI-derived embryos: PGT-A performed in either IVF- or ICSI-derived embryos has similar rates of euploidy and aneuploidy. However, TE biopsies from IVF-derived embryos may result in higher rates of mosaicism compared to ICSI-derived embryos. While using the IVF-derived embryos in PGT cycles, it is advisable to rigorously wash the embryos to remove any attached spermatozoa. Therefore, it is preferable to use ICSI as the method of choice for fertilization in PGT-A cycles to minimize the rate of mosaic results (Palmerola et al. 2019). The optimization and validation of embryo biopsy, vitrification procedures, and genetic testing procedures at the laboratory are crucial in determining the success rate of a PGT cycle (Munné et al. 2020).

9.5 Diagnostic Approaches to Evaluate Embryo Biopsies

The technological evolution of PGT began with screening for aneuploidies by cytogenetic staining using colored probes to visualize chromosomes by fluorescence in situ hybridization (FISH). FISH was used to obtain information on chromosomes 13, 18, 21, X, and Y to mitigate the risk of having live-born babies affected by chromosomal disorders. With growing scientific evidence that PGT improved IVF success rates, testing was quickly expanded to cover more chromosomes. Though chromosome 24 FISH was eventually applied, the technique became cumbersome to be applied to routine practice.

The limitations of FISH led to the discovery of alternate techniques, which eventually led to the development of more efficient molecular diagnostic techniques such as array comparative genomic hybridization (aCGH), quantitative PCR (qPCR), and next-generation sequencing (NGS). These molecular diagnostic techniques not only allowed faster determination of aneuploidy but also expanded the application of PGT to detect segmental aneuploidies, mosaicism, and mutations in embryos.

PGT by next-generation sequencing (NGS) is the latest and most advanced approach to the detection of aneuploidy in single cells. PGT-A by NGS has been demonstrated to have 100% concordance with aCGH (Yang et al. 2015) and to detect segmental changes more precisely in comparison to aCGH. Whole-genome amplification techniques (Table 9.2) in combination with NGS have made PGT possible in samples with DNA as low as 10 ng. The cost-effectiveness, multiplexing, and short processing time have made NGS the most commonly used technique for PGT. The timeline of evolution in PGT techniques is described in Fig. 9.1.

9.6 Work Flow of PGT

- Processing of a biopsy sample begins with the enzymatic extraction of DNA from the embryo biopsy.
- This is followed by whole genome amplification (WGA), which produces enough amount of amplified DNA by PCR. WGA makes it possible to have a sufficient sample for multiple applications and reanalysis, if required.
- Pre-amplification of DNA across the genome is followed by amplification with embryo-specific unique barcodes. These barcodes are unique DNA sequences

Table 9.2 Methods of whole genome amplification from a single cell

Whole genome amplification techniques
Degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR)
Multiple displacement amplification (MDA)
Multiple annealing and looping-based amplification cycles (MALBAC)
Primer extension pre-amplification (PEP)
Single primer isothermal amplification (SPIA)

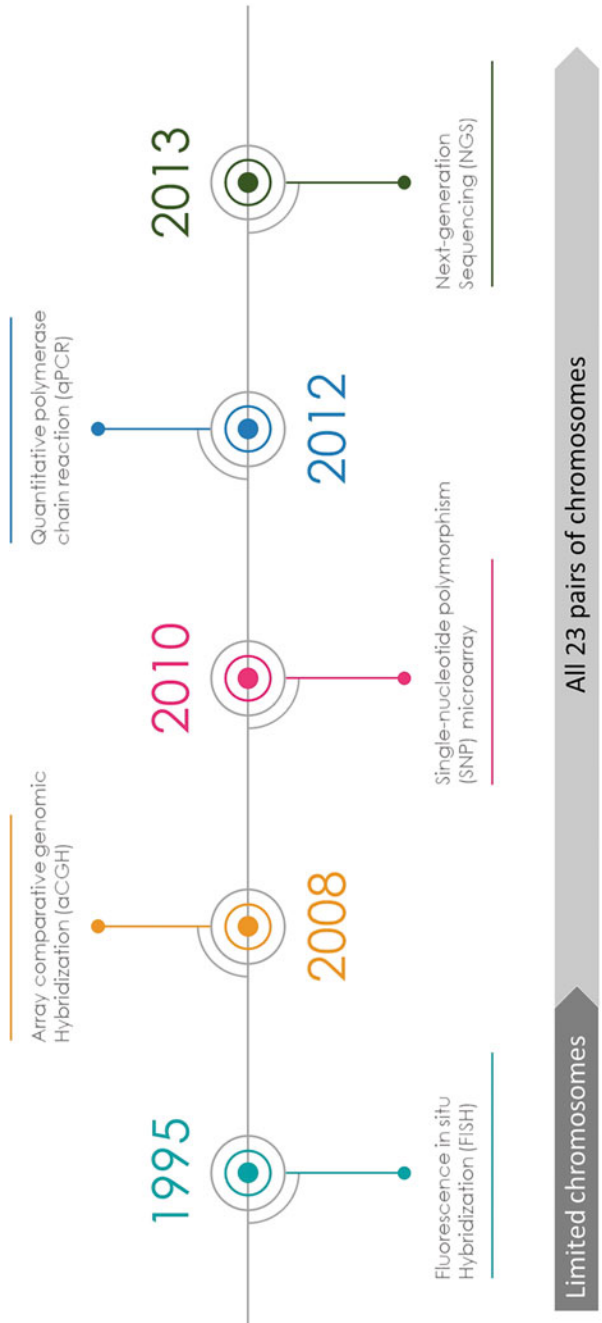


Fig. 9.1 Evolution of PGT techniques

that are adhered to the sample, which allows the pooling of samples and reassigning NGS reads to a specific embryo after sequencing.
- Following sequencing, the data are aligned to reference data on the human genome and accompanied by bioinformatic analysis for whole chromosome and segmental aneuploidies as well as mutations.

9.7 Preimplantation Genetic Testing for Aneuploidies (PGT-A)

The presence of a normal chromosome complement is essential for achieving healthy live births. ART-derived embryos are known to carry chromosomal aneuploidies, which have been implicated as the leading cause of embryonic arrest (Maurer et al. 2015), implantation failure (Lathi et al. 2008), and early miscarriages (Rubio et al. 2005) in patients undergoing ART:

1. Preimplantation genetic testing for aneuploidies (PGT-A) facilitates the selection of chromosomally euploid embryos for transfer.
2. By ruling out the risk of genetic disorders associated with trisomies (e.g., Down syndrome, trisomy 21 or Turner syndrome, monosomy X), significantly reduces the risk of spontaneous miscarriages (it is known that a significant proportion of first-trimester miscarriages are due to loss or gain of genetic material) and can improve the ART cycle outcome by reducing the time to pregnancy.
3. PGT-A has been instrumental in the implementation of elective single embryo transfer (eSET) and reduces the risk of multiple gestations (Chan et al. 2019).

9.8 Incidence of Aneuploidy in Preimplantation Embryos

Over 40% of ART-derived human embryos are known to carry aneuploidies (Fragouli et al. 2014). Cleavage-stage human embryos are known to carry up to 80% of cells bearing whole or segmental chromosome number variations (Chavez et al. 2012; Chow et al. 2014; Mertzanidou et al. 2012), while blastocysts carry a lower incidence of up to 40–50% of aneuploidy (Fragouli et al. 2014).

The etiology of aneuploidy in human embryos has been attributed to meiotic and mitotic errors, fertilization errors, DNA fragmentation, and abnormal cleavage patterns (Tšuiko et al. 2019). Advancing maternal age has been shown to significantly correlate with the increase in aneuploidy in human embryos in both natural and ART-derived pregnancies (Wartosch et al. 2021). Early stages of preimplantation development are engaged in rapid cell proliferation that is associated with relaxed cell cycle checkpoints, low DNA repair capacity, and apoptotic mechanisms, leading to the accumulation of mitotic errors that give rise to segmental aneuploidies. Most modern-day NGS technologies applied in PGT-A can detect segmental aneuploidies down to a resolution of 10–20 Mb. Segmental aneuploidies are just as deleterious as whole chromosome aneuploidies, which are seen in about 16% of ART-derived blastocysts. The detection of segmental aneuploidies is an essential

part of PGT-A, as approximately 6% of the first trimester pregnancy loss is attributed to them.

9.9 The Efficacy of PGT-A

The main purpose of PGT-A has been to improve the success rate of ART cycles; however, the efficacy of PGT-A in achieving improvements in clinical pregnancy rates and live birth rates is a matter of ongoing debate. Chromosomal aneuploidy of meiotic origin is uniformly present in all cells of the preimplantation embryo, meaning a trophectoderm biopsy can reliably represent the chromosomal constitution of the embryo. Aneuploidies of meiotic origin, as commonly seen in advanced maternal age, can be successfully detected with PGT-A and have shown improvement in pregnancy rates (Abhari and Kawwass 2021).

There have been several reports stating the implantation potential of aneuploid embryos (Gleicher et al. 2015; Greco et al. 2015; Patrizio et al. 2019; Victor et al. 2019a, 2019b). The implantation potential of aneuploid embryos has been attributed to the ability of embryos to autocorrect aneuploidy during development beyond the blastocyst stage and the possibility of misdiagnosis due to the phenomenon of mosaicism. Studies addressing the cumulative pregnancy and live birth rates that failed to show an advantage of PGT-A were limited to PGT performed with FISH or array CGH (Cornelisse et al. 2020). Concomitantly, several large-scale studies have proven the efficacy of PGT-A in significantly reducing the risk of miscarriage, particularly in women of advanced age (Sacchi et al. 2019). The main reason for this discordance could be postzygotic mitotic errors that can result in mosaic cell lines. It is also believed that as part of the self-correctional mechanism, the embryo tends to push the abnormal cells into the trophectoderm. This is also further evidenced by the presence of feto-placental mosaicism during postimplantation embryo development, which also limits prenatal screening tests (Grati et al. 2014).

9.10 Embryo Mosaicism

Chromosomal mosaicism refers to the presence of cells having a distinct chromosomal composition in a single sample. Preimplantation embryos are known to carry mosaic cell compositions, as demonstrated by trophectoderm biopsies. Mosaicism can also be present between the trophectoderm and the inner cell mass which has been implicated as the main cause underlying false-positive and false-negative reports in PGT-A. ICM and TE concordance has been reported to be as high as 95–96% by a recent study (Victor et al. 2019a, 2019b).

Mosaic embryos have been shown to have the ability to give rise to healthy live births (Greco et al. 2015); however, they have less potential than completely euploid embryos (Bolton et al. 2016). Therefore, mosaic embryos are chosen for transfer after careful consideration and only in cases where there are no euploid embryos left.

Mosaic embryos can be classified as simple mosaic (mosaicism observed in single chromosomes) or complex mosaic (mosaicism observed across multiple chromosomes). Simple mosaic embryos are further subclassified into low-level mosaicism (mosaicism at 20–40%) and high-level mosaicism (mosaicism at 40–70%).

9.11 Transferring Mosaic Embryos in the Absence of Alternatives

The transfer of mosaic embryos has shown live birth rates in the range of 30–40% and about 20–33% miscarriage rates per mosaic embryo transfer (Abhari and Kawwass 2021). Thw following are according to the PGDIS guidelines:

1. Embryos carrying less than 40% mosaicism have a better clinical outcome than those carrying mosaicism above 40%.
2. Embryos mosaic for trisomies capable of live birth (chromosomes 13, 18, 21, 22), embryos mosaic for trisomies associated with uniparental disomy (chromosomes 14, 15), and embryos mosaic for trisomies associated with intrauterine growth retardation (chromosomes 2, 7, 16) are given a low preference score and hence are not preferred for transfer.
3. It should be noted that mosaic monosomies seem to implant with a similar incidence to mosaic trisomies.
4. Transfer of embryos carrying more than 40% was shown to be associated with poor outcomes.
5. Trophectoderm mosaicism implicating chromosomes 1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 17, 19, and 20 has not been associated with adverse outcomes; therefore, it ranks higher in transfer preference.

However, it is noteworthy that adverse outcomes have been observed when mosaicism is present in the fetus. Therefore, careful counselling of the patient, followed by close monitoring of the pregnancy and invasive prenatal testing by amniocentesis, is advisable in case such embryos are selected for transfer (PGDIS Position Statement on the Transfer of Mosaic Embryos 2019).

Due to technical challenges and the fact that completely healthy human embryos are not readily available for research, the incidence of true mosaicism in human blastocysts is yet to be determined. Since trophectoderm biopsies only represent a fraction of the cells that comprise the blastocyst, more comprehensive methods are being researched to describe the chromosomal status of preimplantation embryos.

9.12 Preimplantation Genetic Testing for Structural Rearrangements

Structural rearrangements comprise reciprocal and Robertsonian translocations, insertional translocations, and inversions. The most common indications for PGT for chromosomal structural rearrangement are familial reciprocal and Robertsonian translocations. Such patients usually have a history of recurrent pregnancy loss or previous pregnancy loss reported with unbalanced translocation in the miscarried tissue. In roughly 5–7% of couples with recurrent spontaneous abortions, a balanced translocation has been discovered in one partner. The incidence of conceiving a fetus with an unbalanced translocation is higher when the female partner is a carrier of a balanced translocation. A higher incidence of segmental aneuploidy is observed in PGT-SR.

Individuals who carry a balanced chromosomal rearrangement involving imprinted genes (e.g., 13, 14 Robertsonian translocation) are at risk of abnormalities related to uniparental disomy, which cannot be excluded by all methods of preimplantation genetic testing. The difference between embryos with a balanced or normal karyotype cannot be distinguished by PGT-SR analysis. Because of these limitations, confirmation of PGT-SR results with CVS or amniocentesis should be offered.

9.13 Preimplantation Genetic Testing for Monogenic Disorders (PGT-M)

Preimplantation genetic testing for monogenic disorders (PGT-M) is performed to assist the conception of pregnancies free from known heritable pathogenic variants carried by one or both biological parents. PGT-M is mainly meant for couples looking for an alternative to invasive prenatal testing, which usually entails difficult decisions on termination of pregnancy in cases where the fetus is diagnosed to be affected. The first reported live-born child through PGT-M was performed to prevent the transmission of X-linked mental retardation (Handyside et al. 1990). Presently, PGT-M is available for a wide range of single-gene disorders. The most commonly tested disorders are listed in Table 9.3. As per guidelines issued by the ESHRE, ASRM, and ESHG, PGT-M is performed only when parents have been established to be affected or are carriers of pathogenic or likely pathogenic genetic variants.

A PGT-M cycle has two phases:

1. The preclinical workup, which includes variant identification and informativity testing
2. The clinical PGT-M, which involves whole genome amplification and variant analysis from the biopsied samples

PGT-M is performed either by direct analysis of the region of interest by targeted bidirectional Sanger sequencing or by indirect linkage-based analysis. For the

Table 9.3 Common single-gene disorders

Autosomal recessive	Autosomal dominant	X linked
Beta thalassemia Sickle cell anemia Cystic fibrosis (CF) Spinal muscular atrophy (SMA) Mucopolysaccharidosis Congenital adrenal hyperplasia Ataxia telangiectasia (AT)	Achondroplasia Neurofibromatosis 1 Marfan syndrome Polycystic kidney disease (PKD) Huntington's disease Tuberous sclerosis Familial hypercholesterolemia Hereditary breast and ovarian cancer (BRCA1/2)	Duchenne muscular dystrophy (DMD Fragile X Hemophilia A Rett syndrome Glucose-6-phosphate dehydrogenase deficiency Charcot-Marie-Tooth disease

indirect testing, first, an informative test is performed, which would require DNA samples from the couple as well as immediate family members. The availability of DNA samples from an affected person in the family (the index case) can help improve the efficacy of PGT. Short tandem repeat (STR) markers are short stretches of DNA, usually measuring about 2–3 base pairs long, which repeat in tandem and vary highly in numbers among individuals, thereby allowing the identification of an individual's unique genetic signature. During the preclinical workup, unique STRs flanking upstream and downstream of the region of interest are identified and used to identify the at-risk haplotype. Once the risk-causing haplotype has been identified, an NGS-based indirect testing strategy may be employed.

The challenge while testing for monogenic disorders is the limited amount of DNA that is available. Therefore, trophectoderm biopsies are always preferred for PGT-M as there is a sufficient amount of the starting material when compared to single-cell amplification. This, in turn, reduces the risk of WGA failure and allele dropout (ADO), in which one of the alleles may fail to amplify during repeated PCR cycles. The risk of ADO has been greatly reduced by advanced whole genome amplification methods that allow efficient amplification of the original sequence, thereby increasing the efficacy of PGT-M.

The application of NGS platforms for PGT-M has made it possible to perform simultaneous assessments on aneuploidies and single-gene mutations. However, NGS-based PGT-M is not cost-effective, unlike direct sequencing. A reduction in sequencing costs in the future would make NGS a more common approach to performing PGT-M.

9.14 Preimplantation HLA Typing

PGT-M may be extended to include compatibility diagnosis of human leukocyte antigen (HLA), which can aid in conceiving a child who can donate cord blood stem cells or bone marrow cells to treat an affected sibling. The extraction of stem cells from an HLA-matched donor is proven to be the best treatment alternative for several genetic disorders such as beta thalassemia, Fanconi's anemia, etc. HLA

matching is performed through the assessment of short tandem repeats (STRs) in the HLA region. PGT for HLA matching alone should only be considered after carefully weighing out the risks and benefits.

The application of HLA matching with PGT-M has been limited by its low success rates. This is mainly due to the low incidence of finding an HLA-matched sibling. Moreover, embryos have to undergo screening for aneuploidies and mutations, which can considerably reduce the number of embryos available for HLA matching. For example, in cases of autosomal recessive conditions such as beta thalassemia, the possibility of finding a mutation-free, HLA-matched embryo is as low as 19% and can drop to 12.5% for autosomal dominant or X-linked diseases. Patients usually have to undergo multiple ART cycles to pool down as many embryos as possible, which is a financially taxing process. Despite its challenges and ethical considerations, this procedure has, however, been successfully implemented in many cases.

9.15 Noninvasive Preimplantation Genetic Testing (NiPGT)

Conventional approaches to PGT require embryo biopsies to be performed by trained staff and an operative protocol conducive to ensuring post-biopsy embryonic survival and sufficient DNA for PGT analysis. It also attracts additional costs in terms of acquiring specialized equipment. Additionally, the possibility of the biopsy procedure compromising embryo freeze-thaw survival led to the exploration of alternate methods to perform PGT. The presence of cell-free embryonic nuclear DNA (cf DNA) found in both blastocoel fluid and spent embryo culture medium (SEM) has made it possible to bypass invasive procedures to investigate embryo aneuploidy status. Noninvasive preimplantation genetic testing (NiPGT) is less labor-intensive and does not require an advanced skill set. In addition, NiPGT is based on the sequencing of cfDNA that is more likely released from both TE and ICM cells and is thus thought to be a better representative of the embryo compared to the TE biopsy alone. At the moment, this technology is available to screen for aneuploidies alone. Indications for NiPGT are the same as that of PGT-A, and it is popular among patients of advanced maternal age, where only 1–2 embryos are available for screening.

The presence of genomic DNA in SEM has been reported as early as days 2–3 of embryonic development. DNA in the SEM is found to be of superior integrity and greater quantity compared with that in the blastocoel fluid, still lower amplification rates and higher ADO rates are observed using the conventional trophectoderm biopsy. Studies assessing the potential of NiPGT-A using SEM have shown promise in fulfilling the clinical requirement of being able to consistently obtain a diagnosis with high informativity rates.

Sampling for NiPGT requires implementation of single embryo culture in about 20 μl of embryo culture medium, beyond days 3/4 of development. Studies have evaluated a number of combinations to optimize culture duration to facilitate successful DNA amplification as well as the highest level of concordance with the

whole embryo. Current literature shows the highest level of concordance is achieved when SEM is collected on day 6 (about 89%) when compared to day 5 (about 68%) of culture. It is also noteworthy that the high sensitivity of NiPGT has been shown only in the SEM samples harvested on day 6 (Lledo et al. 2021). The requirement to culture embryos until day 6 has raised concerns about the unknown impact of extended in vitro culture on embryos.

The reliability of NiPGT strategies, concerning the concordance of genetic results relative to those obtained from whole embryos or biopsy specimens, seems promising but varies widely between published studies, mainly due to culture volume, culture duration, sampling time, analytical techniques employed, and descriptions of concordance rates. However, in most cases, concordance rates fluctuate and are probably considered unacceptable for most clinical applications in their present state. The discordance of results between SEM and embryo biopsy samples may stem from embryonic mosaicism and/or DNA contamination, predominantly of maternal origin (cumulus cells). The potential risk of genetic contamination in SEM is of significant concern, which makes conventional IVF not a feasible fertilization technique for NiPGT. It might result in severe outcomes, potentially leading to the misdiagnosis of embryos. Additionally, the mechanisms underlying the release of embryonic DNA remain unclear. It is crucial to confirm the origin of extraembryonic DNA with accurate data interpretation and to avoid clinical misdiagnosis. The causes underlying the discordance with results from embryo biopsy specimens should be investigated. PGT based upon blastocentesis or SEM samples should, at present, only be considered a screening test at best until further studies prove its efficacy and safety for clinical applications.

9.16 Conclusions and Future Perspective

The rise in the number of ART cycles and the rapid development of potent technologies to assess the chromosomal content of embryos have facilitated the incorporation of PGT into the ART cycles. At present, trophectoderm biopsies are accepted as the best representative of embryonic genetic status. PGT-SR and PGT-M have aided a large group of couples in conceiving healthy babies of their own. These success stories and the support offered by the current medical system are encouraging more couples to accept this direction in their fertility journeys. While novel techniques that aid the noninvasive assessment of embryonic chromosomal content are gaining popularity, their application to a widespread cohort will depend on live birth data, and they are yet to be accepted in routine clinical practice. Appropriate counselling must be offered to patients when new techniques are used for embryo selection in ART cycles.

The emergence of new molecular technologies for embryo selection has positively stimulated the value and use of PGT in ART, although its role in improving the live birth rate is still to be confirmed in prospective randomized trials. The expansion of the application of PGT to assess multifactorial conditions and epigenetic disorders may enable us to further prioritize embryos for transfer and mitigate

the risk of multiple diseases or developmental disorders, even due to DNA methylation changes. Clinical application of genome-wide SNP array and NGS technologies can help in developing better approaches to diagnose and avoid complex diseases.

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Mitochondrial DNA Testing in Assisted Reproduction

10

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Abstract

In this chapter, the authors explain the role of mitochondrial DNA content to determine oocyte quality and implantation potential to increase the pregnancy success rate in couples undergoing infertility treatment. Currently, three companies offer mitochondrial DNA content testing: Igenomix (MitoScore), Progenesis (MitoSure), and Reprogenetics (MitoGrade). This method of genetic testing is on its way to being implemented for infertility workups. Oocytes of higher quality should contain an optimal number of mitochondria that allow for normal fertilization and development of the embryo. Studies have found that once fertilization has occurred, embryos with lower levels of mtDNA have higher rates of implantation. In conclusion, higher mtDNA levels in embryos have been associated with implantation failure, and optimal levels of mitochondria in oocyte are needed for successful fertilization and preimplantation embryo development.

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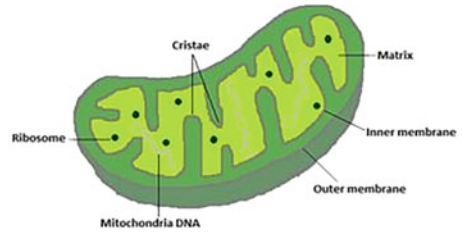
Mitochondrial DNA · Assisted reproduction · Infertility · Embryo quality · Implantation potential · Genetic testing · Reproductive medicine

10.1 Introduction

The mitochondria, as the powerhouses of the cell, are of crucial importance for cellular function. In addition to production of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS), they have several vital roles in the cell (Stimpfel et al. 2017) such as in cellular processes, including calcium (Ca^{2+}) handling and signaling, reactive oxygen species (ROS), apoptotic regulation, and in mediating cellular and organismal aging (Balaban et al. 2005; Dumollard et al. 2007).

During oogenesis, mitochondrial population is amplified. Once the full complement of mitochondria is established in full-grown oocytes, there are no further increases in the population of mitochondria during early development (Dumollard et al. 2007). The content of mitochondria in an embryonic cell can indicate its energy status and can also provide an indication of the amount of its energetic stress. Biogenesis and bioenergetics of mitochondria play an essential role in oocyte maturation and embryonic development (May-Panloup et al. 2005). With age, the mitochondrial functional integrity of oocytes is reduced, resulting in poor oocyte quality, reduction of fertilization rate, impaired embryonic development, and unsuccessful pregnancies (Woods and Tilly 2015). Failure of mitochondrial differentiation, insufficient redistribution of mitochondria, or reduced mitochondrial transcription can lead to impaired fertilization and poor embryo development (Au et al. 2005).

Mammalian mitochondria carry a circular, double-stranded DNA molecule (mtDNA) (Anderson et al. 1981), which is 16.6 kb long, as depicted in Fig. 10.1 and contains 37 genes that code for 24 RNA molecules and 13 polypeptides (Taanman 1999) and are all part of the electron transport chain, which facilitates ATP production (Kim and Seli 2019). Human cells have hundreds to thousands of mitochondria, each having two to ten copies of mtDNA in its matrix. It has been reported that the mitochondrial gene copy number (mtDNA) is correlated with oocyte quality and embryonic development (Collazo et al. 2018). The highest mitochondrial DNA content is found in the mature oocyte, which is directly associated with the oocyte's capacity to support early embryonic development in several species (May-Panloup et al. 2021). Mitochondrial DNA is essential for successful fertilization and serves as a significant marker of oocyte quality that can explain a number of fertilization failures (Santos et al. 2006). Failures in assisted reproductive technology (ART) are attributed to oocyte aging, and the mitochondria play a crucial role in oocyte aging, which is greatly affected by mitochondrial dysfunction (Zhang et al. 2017). Moreover, disorders such as endometriosis, ovarian insufficiency, mtDNA mutations, and aneuploidy of embryos can be related to the



Oocyte:

- Higher number of mitochondria.
- No mtDNA replication.
- Preferred source of energy; pyruvate.
- Production of a lower ATP levels.
  Mitochondrial structure and distribution;
- Primary oocyte: spherical-shaped mitochondria contain dense matrix and few peripheral arched cristae, dispersing throughout the cytoplasm.
- After fertilization: accumulation of mitochondria at the oocyte center, around the pronuclei.

Embryo:

- Lower number of mitochondria.
- No mtDNA replication in the blastocyst stage.
- A significant reduction in total mtDNA copy number before implantation.
  Preferred source of energy;
- Cleavage-stage embryo: pyruvate and lactate.
- Morula/compact embryo: glucose.
- Production of a higher ATP levels in morula and blastocyst embryo.
- Up-regulation of aerobic respiration during blastocyst development and implantation.
  Mitochondrial structure and distribution;
- Early cleavage-stage embryo: elongated mitochondria with transverse cristae.
- Blastomeres in the ICM: spherical mitochondria.
- Blastomeres in the trophectoderm: elongated mitochondria.

Fig. 10.1 Comparison of the mitochondrial structure and function in oocyte and embryo

mtDNA in the oocyte or the embryo (May-Panloup et al. 2005; Xu et al. 2015; May-Panloup et al. 2007; Monnot et al. 2013). For example, a higher mtDNA in euploid embryos is associated with poor implantation potential and could be indicative of decreased metabolic energy during oocyte maturation (Diez-Juan et al. 2015).

In recent years, increased attention has been paid toward the role of the mtDNA in oocyte maturation, embryo development, and the outcome of ARTs. Advances and new technologies for the use of mitochondria and the mtDNA have opened up opportunities for therapeutic improvements, which might increase the success rate for in vitro fertilization (IVF) of oocytes with poor quality (Schatten et al. 2014). In this chapter, we try to give a comprehensive overview of the mtDNA that could help to understand its importance in oocyte maturation and embryo development.

10.2 Mitochondrial Functions in Oocytes and Preimplantation Embryos

In the human female fetus, most oocytes undergo atresia through the apoptotic process, from a maximum of seven million in mid-gestation to approximately two million at birth, falling to 500,000 at puberty (Baker 1972). The mitochondria are involved in follicular atresia (May-Panloup et al. 2016), as an outflowing of cytochrome C from dying mitochondria is an important event in the apoptotic process (Shimizu et al. 1999). High-quality oocytes have a sufficient number of

mitochondria with appropriate quality and function that supply the energy needed for normal fertilization and development of the preimplantation embryo, leading to a successful pregnancy (Woods and Tilly 2015). The primordial oocytes have around 10,000 mitochondria (Haskett 2014), while there are 100,000 mitochondria in mature oocytes (Monnot et al. 2013). Normal blastocysts have a lower number of mitochondria than mature oocytes; this is probably due to the segregation of the original mitochondria into the blastomeres without mitochondrial biogenesis during the fertilization process through the blastocyst stage (John et al. 2010).

ATPs produced by the mitochondria are necessary for oocyte meiotic spindle assembly, chromosome segregation, maturation, fertilization, and preimplantation embryogenesis (Dumollard et al. 2007; Van Blerkom 2011; Bentov et al. 2011). The embryo uses pyruvate or glucose as an energy substrate according to its developmental stage (May-Panloup et al. 2021). Pyruvate, as the final product of aerobic glycolysis (Mayes and Bender 2003), is the preferred source of energy during the early cleavage stages (Martin and Leese 1999). Although the embryo has very low glycolytic ability during the first day or two of development, glycolysis increases sharply after blastocyst formation. The oxygen consumption rate also increases around the time of blastocyst formation (Leese 2012). It has been reported that upregulation of aerobic respiration occurs during blastocyst development and implantation of the embryo (Wilding et al. 2009). Mature oocytes produce lower ATP levels than those observed in morula and blastocyst embryos (Smith et al. 2000). Variabilities in the ATP content can be considered a marker for abnormal oocyte quality, impaired embryo development, and reduced pregnancy rate (Van Blerkom et al. 1995). Furthermore, the ATP produced by mitochondria aids in protein phosphorylation/dephosphorylation, which are regulatory events that are important for the dynamic role centrosomes, and microtubules play in meiotic spindle formation during meiosis I and II (Schatten et al. 2014).

Appropriate management of redox status and ion fluxes is essential for fertilization, oocyte activation, and meiosis completion and development (Dumollard et al. 2007). In this context, the mitochondria play an important role by maintaining calcium oscillations (Kashir et al. 2010). The mitochondrial Ca^{2+} uptake modulates the Ca^{2+} wave pacemaker. Oocytes with low ATP generation due to low mitochondrial and mtDNA content generally are not expected to sustain the Ca^{2+} wave pacemaker and complete meiosis upon fertilization (Tripathi and Chaube 2012). Moreover, Ca^{2+} oscillation triggers the exocytosis of cortical granule enzymes into the perivitelline space to modify the zona pellucida and prevent further sperm entry (polyspermy) (Hojnik and Kovačič 2019). Furthermore, the mitochondria of the oocyte play an important role in regulating the sperm-triggered Ca^{2+} wave pacemaker necessary for zygote activation by acting as a calcium reservoir (Dumollard et al. 2003).

In humans, the primary oocyte has spherical-shaped mitochondria dispersed throughout the cytoplasm that contain a dense matrix and a few peripheral arched cristae (Motta et al. 2000). After fertilization, the mitochondria accumulate at the oocyte center, around the pronuclei. From fertilization to the early stages of embryonic development, the mitochondria are found around the nucleus to provide the

energy required for the formation and fusion of pronuclei (Barnett et al. 1996; Van Blerkom et al. 1998). In a human pronuclear embryo, the cytoplasmic halo is the manifestation of the microtubule-organized translocation of mitochondria and other cytoplasmic components to the perinuclear region of the oocyte (Van Blerkom et al. 2000). The presence of the cytoplasmic halo during fertilization is associated with cell cleavage pattern, blastocyst formation, and ongoing pregnancy rate after a single blastocyst transfer (Ezoe et al. 2020). An elongated mitochondrion with transverse cristae is observed in early cleavage-stage embryos (Motta et al. 2000; Pikó and Matsumoto 1976). As development proceeds, the proportion of mitochondria with these characteristics increases. By the blastulation, blastomeres in the inner cell mass (ICM) contain spherical mitochondria, while those of the trophectoderm contain elongated mitochondria (Motta et al. 2000; Nadijcka and Hillman 1974; Mohr and Trounson 1982; Sathananthan and Trounson 2000; Lees et al. 2017). These morphological changes maximize the surface area available for mitochondrial oxidative phosphorylation (Zick et al. 2009). During mitosis, the mitochondria are randomly distributed between the daughter cells. If blastomeres of the embryo do not receive enough mitochondrial numbers capable of generating ATP, they may become dysfunctional and fragment (Cummins 2004). These differences in both structure and function of the mitochondria may reveal differences in energy demand (Harvey 2019). The rate of oxygen consumption increases toward the morula stage before the increase in mtDNA at the blastula stage (Hashimoto et al. 2017).

It is assumed that insufficient mtDNA could result in abnormal ovulation due to abnormal levels of ATP in the oocyte (John 2014). There are 50,000–1,500,000 copies of mtDNA in the mature oocytes (Monnot et al. 2013). The presence of a minimum threshold of mtDNA at the moment of sperm penetration is essential for successful fertilization and development of human and mouse oocytes (Santos et al. 2006; Wai et al. 2010; Reynier et al. 2001). In a study using mouse model, this minimum threshold has been reported to be approximately 40,000–50,000 mtDNA copies (Wai et al. 2010). However, in humans, this threshold is not yet known (Woods and Tilly 2015). Although the mitochondrial structure and respiratory function develop with embryonic growth, the mtDNA content reduces transiently at the blastula stage (Hashimoto et al. 2017). Before implantation, a significant reduction in the total mtDNA copy number occurs in human embryos, and there is no reported mtDNA replication in the blastocyst stage (Pérez-Sánchez et al. 2020). Mitochondrial DNA replication does not occur until the gastrula stage in diverse species (Taylor and Pikó 1995; Larsson et al. 1998; El Meziane et al. 1989). For example, mtDNA replication in mouse embryos resumes around days 5–6 and lasts until the blastocyst stage (Aiken et al. 2008). There is a positive correlation between the embryonic cleavage rate and blastomere volume with the mtDNA (Murakoshi et al. 2013). Evaluation of mtDNA quantification could be helpful for selection of the best-quality embryos and thus better pregnancy outcomes (Murakoshi et al. 2013). During IVF procedures, embryo implantation is assumed to be closely associated with the mtDNA in cumulus granulosa cells. It is argued that mtDNA quantification in the cumulus granulosa cells could be considered a biomarker for an embryo's implantation potential (Taugourdeau et al. 2019). Figure 10.1 illustrates

the comparison of the mitochondrial structure and function in the oocyte and the embryo.

10.3 The mtDNA Content

Embryo chromosomal status is a current standard for assessing embryo quality for IVF (Zmuidinaite et al. 2021). However, in embryos with normal chromosomal makeup, implantation and pregnancy are still not guaranteed, with about 25–50% of euploidy embryo IVF cycles failing (Wells 2017; Harton et al. 2013). Mitochondrial DNA (mtDNA) level has been proposed as a marker correlated with embryo implantation and pregnancy rates (Kim and Seli 2019). The mitochondria as discussed earlier are vital for successful reproduction, including embryonic development and pregnancy (Babayev and Seli 2015; Wilding et al. 2001), and are vulnerable to environmental and genetic insults (Fragouli et al. 2015; Desquiret-Dumas et al. 2017). Mitochondrial instability may lead to poor oocyte quality and subsequent decline in fertility (May-Panloup et al. 2016). There are different laboratory methods for assessing mtDNA content, including the commercially available assay "MitoScore" and "MitoSure" (Kim and Seli 2019).

Even though mtDNA embryonic content can be measured, there is inconclusive data about the effect of mtDNA levels on successful IVF outcomes (Crosnoe et al. 2013). Lower mtDNA levels have been associated with increased embryo viability. A study completed by Diez-Juan et al. (2015) retrospectively evaluated the link between the mtDNA content of embryos and implantation rate(Diez-Juan et al. 2015). For this, embryos were placed into categories, mtDNA A (MsA), MsB, MsC, or MsD depending on the number of mtDNA copies. MsA had the lowest number of mtDNA copies (<34 copies), MsB was low-middle range (34–52), MsC was high middle range (52–97), and MsD was high range (>97). Results concluded that a lower number of mtDNA copies (and better Ms. categorization) was associated with a higher rate of embryo implantation. MsA was found to implant 59% of the time; contrastingly, MsD implanted 18% of the time and embryos with >60 mtDNA copies did not implant at all (Diez-Juan et al. 2015). Similarly, Fragouli et al. found that embryos with lower mtDNA levels had higher rates of implantation and that there was a threshold of mtDNA levels above which implantation was never observed (Fragouli et al. 2015). High mtDNA levels have thus been associated with implantation failure, while lower mtDNA levels are found to correlate with higher rates of implantation. Moreover, a lower mtDNA is correlated with better embryonic development. Bayram et al. evaluated the relationship between mtDNA and embryonic developmental kinetics/ploidy status. For this, embryos were divided into two groups based on mtDNA amount (low and high mtDNA), and subsequent embryonic development was measured. Results demonstrated a correlation between a lower mtDNA value and earlier embryo development into a blastocyst as well as higher rates of euploidy (Bayram et al. 2019). These findings were congruent with Fagouli et al., who found that there were elevated levels of mtDNA in embryos with aneuploidy, regardless of maternal age (Fragouli et al. 2015).

When assessing only euploid embryos, mtDNA can be used as a marker of implantation potential. Ravichandran et al. found that in euploid embryos, mtDNA may predict pregnancy success(Ravichandran et al. 2017). The authors evaluated chromosomally normal blastocysts for mtDNA levels. After embryo transfer, they discovered that embryos with normal mtDNA levels were more likely to successfully implant and continue on to pregnancy. Embryos with elevated mtDNA levels had lower rates of implantation. None of the embryos with elevated mtDNA developed into a successful pregnancy (Ravichandran et al. 2017). Fragouli et al. (2017) also evaluated the association between mtDNA levels and implantation rates of blastocysts. All blastocysts studied were euploid with normal morphological features. All blastocysts involved in a successful pregnancy had low or normal levels of mtDNA. Blastocysts with the same chromosomal and morphological features but with high levels of mtDNA were associated with unsuccessful pregnancy (Fragouli et al. 2017). Lower mtDNA levels may thus indicate a higher chance of euploid embryo implantation.

10.4 Evidence Against the Utility of mtDNA Testing

Though there is significant research supporting the use of mtDNA testing in the embryo selection process (Diez-Juan et al. 2015), there is also compelling evidence that mtDNA levels may not predict successful implantation (Treff et al. 2017; Klimczak et al. 2018). For example, a study from Treff et al. (2017) suggested that in double embryo transfer, mtDNA levels are not associated with the likelihood of implantation(Treff et al. 2017). This argument was based on a retrospective evaluation on 187 patients to see if mtDNA could be used to determine which embryos will, and which will not, successfully implant during a double embryo transfer (DET). Mitochondrial DNA values were collected prior to implantation. Two embryos (one male and one female) were transferred. After the DET, any pregnancy with a singleton gestation was included. The sex of the successfully implanted embryo was used to reference the embryo's mtDNA level. Then, the mtDNA levels for successful and unsuccessful implantations were compared. Results suggested no significant correlation between mtDNA levels and the rate of embryo implantation (Treff et al. 2017).

In addition, Fragouli et al. (2015) and his colleagues also reported that mtDNA content was not predictive of implantation or pregnancy rates in euploid embryos and that elevated mtDNA was associated with lower-quality embryos (grade 3, poor quality) rather than mid- or high-quality embryos. However, this association was not found if all embryos were euploid. Similar to this, Klimczak et al. (2018) found that in euploid embryos, there was no association between mtDNA levels and implantation/pregnancy rates (Klimczak et al. 2018). A case report by Bayram et al. (2017) also documented two instances of discordance between mtDNA content and embryonic development (Bayram et al. 2017). Together, these studies suggest that in embryos that are euploid, mtDNA content may not be a good marker of implantation potential.

The question that arises is, does the method of interpretation of mtDNA alter its value as an implantation marker? For example, when mtDNA values were interpreted in the context of nuclear DNA value, no major mtDNA level differences between blastocysts were observed. In this context, Victor et al. (2017) assessed the mtDNA values of embryos when corrected for nuclear DNA value. To achieve this correction, each embryo mtDNA value was divided by the embryo nuclear DNA value. After normalization, the amount of blastocyst mtDNA was compared between embryos based on different classifications. The authors compared maternal age (young maternal vs. advanced maternal), implantation success (successful vs. unsuccessful implantation), and chromosomal status (aneuploidy vs. euploidy). The results suggested no significant differences in mtDNA levels between the considered classifications (Victor et al. 2017), thereby showing that mtDNA, when corrected for nuclear DNA, is not associated with maternal age, implantation rates, or embryo chromosomal status. Table 10.1 summarizes studies for/against the utility of mtDNA testing.

10.5 Mitochondrial DNA Content in Fresh and Frozen Embryos

There has been limited recorded difference in mtDNA content between fresh and frozen embryos. In a study conducted by Sayed et al. (2021), the mitochondrial content was found to be higher in fresh embryos compared to frozen embryos, though it was not statistically significant (Sayed et al. 2021) (Table 10.2). This process leads to decoupling of the inner mitochondrial membrane and a decrease in ATP production known as the hypothermia-hypoxia phenomenon. As a result, there is an increase in oxygen level and the formation of free radicals that cause damage to the embryo. This study also showed that the pregnancy rate for the frozen egg transfer group was not significantly higher than for the frozen egg transfer group (Sayed et al. 2021). Together, the documented research so far suggests that mtDNA content is not significantly different between fresh and frozen embryos that have undergone division properly, and therefore, it should have no effect on embryo quality and IVF success.

10.6 Mitochondrial DNA Content and Its Relation to Recurrent Implantation Failure (RIF)

Failure of the embryo to implant is one of the most common causes of an unsuccessful pregnancy. Recurrent implantation failure occurs when a couple has had three failed attempts of ART with embryos that were considered good quality (Carp et al. 2004) (Table 10.2). Although it is not an independent factor, the mtDNA content can potentially be utilized as a predictive measure for selecting the highest-quality embryos for implantation. Many studies have proven that euploid embryos with good morphology and a normal/low mtDNA content have a higher pregnancy rate than euploid embryos with good morphology and a high mtDNA content. Thus,

Table 10.1 Studies for/against the utility of mtDNA testing

First author	Publication year	Study design	Study population	Results
Diez-Juan	2015	Retrospective analysis	270 female patients, IVF clinic	Lower mtDNA levels, associated with increased embryo viability
Fragouli	2015	Prospective observational study	379 embryos, IVF clinic	Lower mtDNA levels, associated with increased embryo viability
Fragouli	2017	Prospective observational study	174 couples with 199 blastocysts, IVF center	In morphologically and chromosomally normal blastocysts, lower rates of mtDNA were associated with successful pregnancy when compared to higher rates of mtDNA
Treff	2017	Retrospective study	187 female patients undergoing DET with one female and one male embryo, IVF center	mtDNA level not associated with implantation rates in DET
Victor	2017	Retrospective analysis	259 female patients and their 1,396 embryos, IVF center	mtDNA is not associated with material age, embryo implantation, or embryo
Bayram	2017	Case report	Two female patients aged 40 and 45 at IVF center	Case report of two patients with embryos with normal ploidy status and high levels of mtDNA
Ravichandran	2017	Retrospective study	1505 blastocysts, all euploidy, IVF center	In morphologically and chromosomally normal blastocysts, lower rates of mtDNA were associated with successful implantation/ pregnancy when compared to morphologically and chromosomally normal blastocysts with high rates of mtDNA

(continued)

Table 10.1 (continued)

First author	Publication year	Study design	Study population	Results
Bayram	2019	Retrospective study	375 blastomeres, IVF clinic	Lower mtDNA associated with better embryonic development
Klimczak	2018	Retrospective chart review	Female patients ages 31–38, IVF center	mtDNA levels were associated with embryo implantation or pregnancy rates

mtDNA content alone (i.e., without taking embryo morphology into consideration) could be used as a biomarker for successful implantation. Higher mtDNA content is correlated with decreased rates of implantation; as mtDNA content increases, the chance of a well-developed embryo decreases leading to a higher chance of implantation failure. In a study done by Eker et al., it was established that patients with RIF had a higher mtDNA content in endometrial tissues than patients with normal pregnancies. The study evaluated mtDNA amplification results and found that higher mtDNA content in the group of patients with RIF may be a result of increased oxidative stress in the endometrial tissues. This could result in decreased endometrial receptivity and implantation failure. As a result, mtDNA content could be used as a prediction of RIF before an IVF treatment is started.

10.7 Mitochondrial DNA Content and Its Relation to Recurrent Pregnancy Loss

An important part of ART success is the ultimate birth of a live baby. A miscarriage is classified as pregnancy loss before 20 weeks of gestation (Carp et al. 2004). ART success is the birth of a live baby after 26 weeks of gestation. Recurrent pregnancy loss is a common problem with minimal explanation for couples undergoing infertility treatment (El Hachem et al. 2017). Limited studies have been conducted in the context of association between mtDNA content and recurrent pregnancy loss; the available information suggest that there is no significant difference in mtDNA content between blastocysts that resulted in miscarriage and blastocysts that resulted in live births (Zhou et al. 2021) (Table 10.2).

10.8 Mitochondrial DNA Content and Its Relation to Embryo Fragmentation

Uneven division of the cells of the embryo results in embryo fragmentation(Hazlett n.d.). Embryo fragmentation is currently used as a marker for assessing embryo quality (Zhang et al. 2021). mtDNA content is expected to be an indicator of high

Table 10.2 Research studies related to mitochondrial content quantity and its application in improving pregnancy outcomes

First author	Publication year	Study design	Study population	Results
Elpida Fragouli	2015	Independent blinded prospective study	203 normal blastocysts and 99 aneuploid blastocysts	Chromosomally abnormal blastocysts tended to contain significantly larger amounts of mtDNA compared to those which were characterized as being euploid (Fragouli et al. 2015)
Antonio Diez-Juan	2015	Retrospective analysis	Single-embryo transfer in 270 patients	A high mtDNA copy number in euploid embryos is indicative of lower embryo viability and implantation
Elpida Fragouli	2017	Blinded-prospective nonselection study	199 chromosomally normal blastocysts	(Fragouli et al. 2017) 142- successful implantation 57- failed to implant 9- unusually high mtDNA content 121- ongoing pregnancies 11- biochemical pregnancies (implantation occurred but resulted in a miscarriage) 10- spontaneous miscarriages
K. Ravichandran	2017	Blinded retrospective study	1505 euploid blastocysts	The difference between the implantation rates (IRs) for embryos with normal and elevated mtDNA levels was highly significant ($P < 0.0001$)
Amber M. Klimczak	2018	Retrospective chart review	1510 blastocyst biopsies of grade 1 (high), grade 2 (mid), and grade 3 (poor) quality	Embryos with higher mtDNA content were found to be of poorer quality (grade 3) relative to grades 1 and 2. mtDNA best predicted lowest and

(continued)

Table 10.2 (continued)

First author	Publication year	Study design	Study population	Results
				highest grades but not mid-grade embryos
Jie Wang	2021	Retrospective analysis	2001 blastocysts	mtDNA content was higher in aneuploid blastocysts than the euploid blastocysts. Higher quality trophoblast was associated with lower mtDNA content. Euploid blastocysts with higher mtDNA content had a lower chance to implant
Ghadir A Sayed	2021	Prospective study	100 PCOS patients and 33 healthy women as a control group	In fresh embryo culture media, mtDNA/gDNA ratio is higher, though not statistically significant, in comparison with frozen embryo (Sayed et al. 2021)
Qing Zhang	2021	Prospective study	223 embryos from	mtDNA/gDNA ratio increased overall with an increase in embryo fragment content but did not differ significantly between high-, medium-, and low-quality embryos (Zhang et al. 2021)

PCOS polycystic ovary syndrome, *gDNA* genomic deoxyribonucleic acid

degree of embryo fragmentation. In this context, study from Zhang et al. (2021) reported that mtDNA content increases as the degree of fragmentation increases and embryos with less fragmentation had a lower mtDNA and were more likely to develop to the blastocyst stage. Previous reports described that fragmentation indicated poor embryo development. Increased fragmentation is typically associated with genetic abnormalities and embryonic defects. Embryo fragmentation could be as a result of apoptotic activity (Zhang et al. 2021). Although the patterns were interesting, the results were not statistically significant (Zhang et al. 2021). In

summary, there is no direct evidence suggesting a correlation between mtDNA and embryo fragmentation.

10.9 Mitochondrial DNA Content and Its Relation to Ploidy Status

An essential part of fertility treatments is preimplantation genetic testing to rule out chromosomal abnormalities and avoid transferring embryos with genetic and chromosomal abnormalities. An embryo that is euploid contains 46 chromosomes (23 pairs) and is considered normal, while an embryo that is aneuploid has a gain or loss of chromosomes and is considered abnormal (Potapova et al. 2013). Several studies have indicated that aneuploid blastocysts contain high mtDNA content compared to euploid blastocysts (Fragouli et al. 2015). However, there are studies which counter this argument suggesting that there is no significant difference in mtDNA content between euploid and aneuploid blastomeres (Lee et al. 2019).

10.10 Mitochondria Transfer as a Tool for Improvement of Embryo Quality and Infertility Treatment

Currently, the transfer of mitochondria has several clinical applications including the prevention of mtDNA disease, aging oocytes, and diabetic mothers (Reznichenko et al. 2016). It is known that aging oocytes contribute to aneuploidies and infertility and that mitochondrial disease is passed to the child from the mother (Reznichenko et al. 2016). The process of mitochondrial transfer can preclude a mother from passing down mtDNA disease and help women with aging oocytes and/or diabetes conceive a healthy child, though the child will be partially genetically unrelated to the mother (Reznichenko et al. 2016). Considering these findings, It is argued that mitochondrial transfer could potentially be used in infertility treatments for cytoplasmic defects as a result of aging (Reznichenko et al. 2016). There are several techniques used in mitochondrial transfer including pronuclear transfer, spindle transfer, cytoplasmic transfer, and blastomere transfer (Reznichenko et al. 2016). One concern of using mitochondrial transfer is mitochondrial-nuclear genome incompatibility, which could have deleterious effects on the metabolism of cells and can potentially result in a child with abnormalities. Appropriate interactions between nuclear and mitochondrial genes are important for metabolism in cells (Reznichenko et al. 2016). Although the mitochondrial genome is highly conserved, mitochondrial donors should be from the same ethnic group as the recipient to avoid mitochondrial-nuclear genome incompatibility (Lee et al. 2019). However, further research is necessary to ensure the safety and efficacy of mitochondrial transfer.

10.11 Mitochondrial DNA Content as a Biomarker of Implantation Potential

Over the last few years, assisted reproductive technologies (ART) have advanced significantly in their ability to select an embryo for IVF generation based on its reproductive potential (Ogur et al. 2019). Initially, only embryologic morphology and cleavage rates were used to choose acceptable embryos for transfer; however, while these measures improved outcomes, the majority of transferred embryos still failed to implant and produce ongoing pregnancies (Kim and Seli 2019). On the other hand, multiple pregnancies are one of the most common and serious complications of ART, and they increase the risk of adverse obstetric and perinatal outcomes. Increasing the number of transferred embryos may lead to an increased rate of multiple pregnancies (Ogur et al. 2019). The ability to transfer only euploid embryos led to a substantial increase in live birth rates with the advent of preimplantation genetic testing for aneuploidy (PGT-A) (Kim and Seli 2019).

Preimplantation genetic testing for aneuploidies (PGT-A) was first introduced in 1993 for the purpose of selecting euploid embryos for transfer and increase pregnancy success in assisted reproductive technology (Greco et al. 2020). At this time, screening was performed using fluorescence in situ hybridization (FISH), but this only allowed a few chromosomes to be analyzed. Eventually, various techniques were developed to allow for the determination of embryo ploidy status with a complete chromosomal panel. These included array comparative genomic hybridization (aCGH), next-generation sequencing (NGS), and real-time quantitative polymerase chain reaction (rt-PCR) (Greco et al. 2020). With these techniques, biopsies can be collected via sampling polar bodies, blastomeres, and trophectoderm.

Today, NGS is the most used technique for preimplantation genetic testing. First, whole genome amplification is conducted, and then, different samples are taken and labelled with specific sequences. Each sequence is then compared to a reference human genome and ran through a software that can identify copy number variations and large deletions or duplications (Greco et al. 2020).

Once the embryo has undergone the proper screening via subjective morphology of the embryo, evaluation of embryonic growth, and PGT-A testing, the last step in deciding which embryo has the greatest chance for successful implantation can be determined through mitochondrial DNA testing (Paulson 2022). One of the benefits of mitochondrial DNA testing is that the sample taken for preimplantation genetic testing (PGT) can be used for mtDNA testing, thus eliminating the need for another biopsy. The biopsy is collected on the third or fifth day of embryonic development, and the PGT test is performed in the blastocyst stage (fifth or sixth day). The embryos that will undergo testing are then frozen using the vitrification technique, an alternative approach to cryopreservation that enables hydrated living cells to be cooled to cryogenic temperatures in the absence of ice (Fahy and Wowk 2015). Next, the mtDNA testing is performed via next-generation sequencing (NGS). The results are generated by measuring the ratio of mitochondrial DNA to nuclear DNA.

Despite these breakthroughs, euploid embryo selection and subsequent transfer may not always result in continuing pregnancies, demonstrating that embryonic viability is more than just chromosomal normalcy (Kim and Seli 2019; Bayram et al. 2017). A woman's maternal genes affect her fertility to a large extent, and mtDNA plays a significant role in this process from fertilization to oocyte maturation and embryonic development (Babayev and Seli 2015; Klimczak et al. 2018). As discussed earlier, though there are contradictions but a considerable consensus is, the mitochondria could be employed as biomarkers for evaluating embryo quality and ART approach selection (Babayev and Seli 2015). The amount of mtDNA in a chromosomally normal embryo is linked to its energy level, which influences the embryo's capacity to implant in the uterus. Mitochondrial function, mtDNA gene expression, and energy are all required for a high-quality embryo to undergo critical cellular divisions. According to several early studies, higher mtDNA concentration in an embryo signifies a low level of energy and, as a result, a decreased possibility of implantation (Bayram et al. 2017; Babayev and Seli 2015).

10.12 mtDNA Testing in Clinics

Currently, there are three companies, Igenomix, Progenesis, and Reprogenetics that have designed a test to quantify mitochondrial content in cells as a way for mtDNA testing. The tests are known as MitoScore (Igenomix), MitoSure (Progenesis), and MitoGrade (Reprogenetics (Paulson 2022)). MitoSure/MitoScore test's predictive value is presently under investigation. It is used to determine an embryo's energy state. Mitochondrial DNA content is claimed to identify embryos with the highest implantation probabilities, which are thus more likely to result in a successful pregnancy through IVF. The incorporation of the mtDNA copy number (MitoScore/MitoSure/MitoGrade) into standard genetic analysis is anticipated to assist IVF clinics in selecting euploid embryos with higher implantation potential (Diez-Juan et al. 2015). However, the concept of mitochondrial function as a parameter for selecting viable embryos to produce the highest implantation success rates is still understudied and requires large-scale validation.

10.13 Conclusions and Future Perspective

Genetic testing is crucial in exploring the causes of infertility and assessing couples who are at risk of transmitting genetic defects to their offspring. The mitochondria and their genome have key roles in oogenesis and early preimplantation development. The role of the mitochondria in the regulation of oocyte maturation and embryo development goes beyond their role in ATP production to provide needed energy. The mitochondria, as dynamic organelles, move to cell-specific regions and communicate with other cellular organelles, which affects signaling cascades, chromosome segregation, and cytokinesis and lineage specification (Harvey 2019). Failure of mitochondrial differentiation, insufficient redistribution of mitochondria,

or reduced mitochondrial transcription can lead to impaired fertilization and poor embryo development (Au et al. 2005). MtDNA is necessary for successful fertilization and can be considered a marker of oocyte quality, explaining several fertilization failures.

Since optimal mitochondrial function is essential for oocyte maturation, fertilization, and embryo development, improvement in mitochondrial function via the use of small molecules or mitochondrial transfer might have an impact on fertility outcomes (Babayev and Seli 2015). The autologous mitochondrial transfer promises the potential to enhance energy and improve IVF success, and the field of ART has been recently inclining toward finding biomarkers predictive of implantation potential in order to increase the pregnancy success rate (Cozzolino et al. 2019). Mitochondrial-focused diagnostics and interventions may play a significant role in providing new treatments for infertility in the future (Fragouli and Wells 2015); however, the widespread acceptability of mtDNA content still warrants for more research and validation. Future testing methods for embryo viability and implantation success may include assessing epigenetic markers in addition to the mtDNA testing. Currently, chromatin rearrangement and DNA methylation are being investigated in association with embryo quality (Poli et al. 2019).

Because of this, genetic testing in reproductive medicine is necessary to determine the cause of infertility. Genetic testing's role in identifying carriers of inherited diseases and planning antenatal testing indicates its importance in the science of reproduction (Cariati et al. 2019). Based on the rate of genetic disorders that are associated with infertility, genetic testing can accommodate most diagnoses of disorders that may be responsible. Since gametes with genetic or chromosomal discrepancies reduce the possibility of pregnancy occurring, preconception screening is crucial. Because of ART, many of the foreseen difficulties can be avoided and genetic tests, thus, have a crucial impact on monitoring the possibility of mutation transmissions.

While rare, it is possible that a genetic test may miss a small mutation in a gene or chromosome. Additionally, for some conditions, even if a chromosomal discrepancy is found, it is not always possible to tell how severe the affliction would be. This is due to the reason that genetic screenings only target specific variables. As such, there are several components of molecular genetic testing that are continuing to be developed. Currently, the diagnostic pathway and effectiveness of genetic testing for infertility suffer from the fact that only few variables are assessed through specific molecular procedures (https://medlineplus.gov/genetics/understanding/testing/riskslimitations/ n.d.). However, recent developments in sequencing technologies have made it possible to compact several tests, thereby cutting costs and time. However, infertility management is undergoing important transformations that will take the concept of preconception screen from a reactive to a preventative approach. Therefore, the future of genetic testing will allow for an integrated diagnosis and treatment that may benefit many individuals (Cariati et al. 2019). This demonstrates that genetic testing is still a crucial component in fertility and reproductive success, which has been proven through the testing that is available for hundreds of inherited diseases.

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Genetic Testing for Endometrial Receptivity 11

Anshita Sharma and Rajender Singh

Abstract

Endometrial receptivity is a gateway to a successful pregnancy. Emerging evidence has proven the crucial role of endometrial receptivity in blastocyst adhesion and embryonic development, yet its establishment and receptivity are not fully understood. The endometrium is a hormonally regulated tissue that is subjected to variations due to a variety of underlying factors. Understanding the molecular basis and genetics behind endometrial receptivity might pave the way to better diagnostics and therapy in infertility management. In this chapter, we will gain insights into the significance of endometrial receptivity in implantation and genetic testing methods to test endometrial receptivity. Special attention is paid to highlight the complexity of endometrial receptivity, the genetic tests employed to predict endometrial receptivity, and the future possibilities for personalized medicine in this area.

Keywords

Endometrial receptivity · Infertility · ERA · Genetic testing · Reproductive medicine · Assisted reproduction · Embryo transfer

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11.1 Introduction

The endometrium is a mucosal multilayered lining of the uterus, known to offer the floor and nourishment for crucial implantation of the blastocyst due to its proliferative ability and communication with hormones, primarily estrogen and progesterone, as they modulate the endometrial development and subsequently sustain the pregnancy. Changes in the endometrial parameters, such as thickness and volume, might hamper the chances of successful implantation and hence should primarily be checked before selecting the implantation window. If any disruption is faced by the endometrium, the adhesion and implantation get negatively affected. The elements deciding the endometrial receptivity are not fully understood, but a lot of factors affect it. Recurrent implantation failure has been linked with variations in endometrial receptivity (Ruiz-Alonso et al. 2013). The underlying reasons for poor receptivity are not well understood yet, but causes such as hormonal breakouts, inflammatory events, thickness of the endometrium, polyp formation, and immunological disorders are under investigation.

11.2 Endometrial Receptivity: The Unsung Hero

Implantation is a critical step in human reproduction and requires the timely arrival of a viable blastocyst to settle in the endometrium for its success. If pregnancy does not occur, the corpus luteum will cease the synthesis of progesterone along with estrogen, marking the regression of corpus luteum itself, and the onset of periodic menstruation (Critchley et al. 2020). If the endometrium and embryo are healthy, pregnancy is established. Pregnancy is always supported when the endometrium is receptive, which occurs for a defined period of time. Endometrial receptivity is essential to understand as the magnitude and type of abnormality may lead to implantation failure, miscarriages, or preeclampsia, thus contributing to infertility (Aghajanova et al. 2008). Governed by ovarian steroid hormones and cAMP signaling, the onset of the window of implantation (WOI) is marked by the decidualization of endometrial stromal cells that occurs during the mid-secretory phase, which is limited to 48 hours starting around the seventh day after the LH surge (Okada et al. 2018). WOI is attributed to a spatially and temporally determined period of trophoblast-endometrial interactions (Wilcox et al. 1999). Endometrial receptivity is crucial for implantation and serves as a checkpoint determining the success of IVF and assisted reproduction.

11.3 Metabolic and Immunological Determinants of Endometrial Receptivity

It has been suggested that obesity, PCOS, and other metabolic disorders impact the alteration of endometrial receptivity (Bellver et al. 2021). A number of cytokines are involved in both endometrial receptivity and embryo development, which are

influenced by infection and inflammation (Granot et al. 2012). The uterine microbiome also plays a significant role in determining endometrial health by getting involved in generating the immune response (Benner et al. 2018). The expression of toll-like receptors (TLRs) is significant in the reproductive tract. Upon pathogen-derived immune response, TLRs get engaged and drive the expression of a number of cytokines, including tumor necrosis factor alpha (TNFA), interleukin 6 (IL6), granulocyte-macrophage colony-stimulating factor (GMSIF), granulocyte-colony-stimulating factor (GSCF), and interleukin 1B (IL1B). The well-known characterization is the binding of TLR4 to a gram-negative mimetic lipopolysaccharide, which initiates a cytokine-induced inflammatory cascade (Ciesielska et al. 2021). These pathways affect endometrial receptivity significantly.

11.4 Molecular Signaling in Endometrial Receptivity

Advancements in genomics and molecular biology have provided significant clues about factors influencing endometrial receptivity. Genes such as leukemia inhibitory factor, homeobox genes A, pinopodes, αvβ3-integrin, and intercellular junction have been implicated in endometrial receptivity (Xu et al. 2012). αvβ3- integrin is reported to be overexpressed in the endometrium of fertile as well as pregnant women (Elnaggar et al. 2017). Homeobox genes increase in the endometrium during the mid-luteal phase and are maintained by progesterone and estrogen levels (Ashary et al. 2020). Hox signaling demonstrates a significant association with endometriosis, PCOS, and leiomyomas (Taylor et al. 1999). Hox genes, when upregulated, increase pinopode number and regulate β3 integrin (Du and Taylor 2016). HOXA10 is a potential marker for endometrial receptivity (Xu et al. 2014). Pinopodes can be used as markers of endometrial receptivity (Nikas 1999). As they can be functional due to their appearance on days 19, 20, and 21 of the 28-day cycle, but the application of scanning electron microscope limits their detection.

VEGF and TNFα are essential for endometrial secretion and, hence, are the key players in embryo implantation. The downregulation of endothelin 3 and a putative progesterone binding protein-PR membrane component 1 (PGRMC1) have also been found to be relevant in endometrial receptivity (Schuster et al. 2010). FoxO1 is also a well-known marker because of its role in the decidualization of the endometrial stromal cells (Adiguzel and Celik-Ozenci 2021). The overexpression of IGFBP-1, CPE1-R, Dkk-1, and ApoD mRNAs during decidualization of stromal cells in vitro aligns well with the results obtained from a microarray analysis (Kao et al. 2002). BMP signaling pathway plays a vital role in endometrial receptivity and implantation through ACVR2A-SMAD1/SMAD5 (Monsivais et al. 2021). The inflammatory events linked with endometriosis include a sudden rise in IL-6 and IL-7, activating Cox-2 signaling and prostaglandin pathways. These changes elevate the KRAS pathway (Yoo et al. 2017), phosphorylate STAT3, and affect proteins that further downregulate STAT3, which expresses usually only at menstruation (Yoo et al. 2016). Such broad changes are responsible for aromatase expression and shift

in hormonal balance. Aromatase is particularly expressed in patients with endometriosis or endometrial carcinoma (Bulun et al. 2005).

11.5 Gene Expression Alterations in Endometrial Receptivity

RT-PCR validated results suggested that 370 genes decreased by 2–100-fold, while 323 genes were upregulated 2–45-fold during the transition of the human endometrium from a pre-receptive to a receptive state (Carson et al. 2002). Another such biomarker of receptive endometrium is glycodelin, which increases in the ovulatory cycle and decreases in the uterine fluid of infertile women. Glycodelin is mainly secreted by the endometrial epithelia at the luteal phase (Skrzypczak et al. 2005). The role of hormones is indisputable in the cyclic patterns. The study of the dynamicity and the cyclical pattern of endometrial receptors (ERa, ERb, PRA, and PRB) is crucial in investigating the factors responsible for endometrial receptivity. With the evolution of microarray technologies, identification of novel genes and pathways has become easier. Haouzi et al. (2009) introduced the concept of genetic biomarkers of ER. They validated the expression levels of five genes specifically modulated during the WOI. Out of these five, laminin β3 (LAMB3), angiopoietin-like 1 (ANGPTL1), prokineticin 1 (PROK1), microfibrillar-associated protein 5 (MFAP5), and nuclear-localized factor 2 (NLF2) rise in the mid-secretory phase. All the genes are essentially involved in angiogenesis and extracellular communication. Integrin testing has recently opened the door to multidimensional assessment of histology and biochemical integrity (Lessey et al. 1992).

11.6 miRNAs: Important Players in Endometrial Receptivity

miRNAs regulate the expression of protein-coding genes. Changes in miRNAs appear much earlier than changes in mRNAs, and hence, they can also be utilized as markers of endometrial receptivity. Studies on miRNAs have shown that miR-183-5p (Akbar et al. 2020) and miR-145 (X. Liu et al. 2020) can be adopted as biomarkers because of their significant roles in endometrial receptivity and embryo implantation. Some of these play critical roles in embryo implantation; for example, miR-101 and mir-199a contribute by targeting Cox-2 gene (Chakrabarty et al. 2007) and miR-193 (Li et al. 2014) by targeting growth factor receptor-bound protein 7 (GRB7) and downregulating HOX A10. miR-519d (Xie et al. 2014) and miR-155 (Dai et al. 2012) act as downregulators of trophoblast proliferation and migration during implantation. miRNAs belonging to the families miR-23, miR-30, miR-200, and miR-183 have a regulatory effect on Wnt signaling. Let-7a and miR-200c influence endometrial receptivity via suppressing canonical Wnt signaling and increasing receptivity in the human uterus. Upregulation of miR-27a, miR-152, and miR-155 in the endometrium and serum of women with recurrent implantation failure (RIF) has been observed (Drissennek et al. 2020). MicroRNAs can be further investigated as therapeutic targets to detect endometrial receptivity in human fertility

treatment (Shekibi et al. 2022). The utility of miRNAs as diagnostic markers for endometrial receptivity has not yet been fully explored. miRNAs can be considered emerging biomarkers for assessing endometrial receptivity (Altmäe et al. 2013; Sehring et al. 2022).

11.7 Classical Methods of Evaluating Endometrial Receptivity

An endometrial receptivity scoring system was needed ever since assisted reproduction became popular. Noyes et al. in the 1950s studied biopsy samples to evaluate endometrial development and receptivity and provided information on the morphological features of the endometrium at different phases of the menstrual cycle (Noyes et al. 1950). It commends morphological factors such as pseudostratification of nuclei, basal cell vacuolation, secretion, stromal mitosis and edema, pseudodecidual reactions, and leukocytic infiltration (Ludwig 1982). Research has suggested performing the biopsy during the mid-luteal phase or the late luteal phase. Assessment in the late luteal phase is accompanied by the detection of the molecular changes in the endometrium, such as adhesion and migration of trophoblast cells of the implanting embryo due to alterations in estrogen and progesterone levels (Castelbaum et al. 1994). These studies confirm ovulation and the maturation of the endometrium but suffer from limitations such as invasive procedures, expertise dependency, sample collection, and cultural requirements. Biopsy timing at the window of implantation is crucial and controversial in humans, which suggests the need for robust markers of endometrial receptivity and successful implantation. There are clinical tools such as ReceptivaDx™, which are designed to evaluate BCL6 concentration in a biopsy sample of the endometrium at 7–10 days after ovulation to evaluate the inflammatory conditions on the uterine lining for embryo implantation. If BCL6 is high, there is a high chance of endometriosis, affecting fecundity (Almquist et al. 2017). However, an endometrial biopsy is no longer accepted because of its high cost, invasiveness, requirement of precise cycle timing for proper interpretation, and inability to differentiate between fertile and infertile women.

This was followed by the development of noninvasive methods, such as ultrasound. Markers such as endometrial thickness, endometrial echo, endometrial wave-like activity, and endometrial blood flow give an indication of endometrial receptivity using ultrasound. 2D transvaginal ultrasonography is currently widely used to measure endometrial thickness and monitor endometrial growth during the menstrual cycle. Though ultrasound precisely assesses the endometrium during IVF cycles, its manual interpretation might suggest false-positive results for receptive state. The sonographic parameters might predict uterine receptivity but still lack specificity, and so the ideal method to predict endometrial receptivity by a noninvasive method has yet to be established. With the advent and evolution of three-dimensional (3D) ultrasound, we now stand at a new threshold in noninvasive diagnosis (Khan et al. 2016). Three-dimensional power Doppler ultrasound predicts implantation rate in IVF cycles (Singh et al. 2011), as it analyzes the whole endometrial and sub-endometrial vascularization, but the findings of these methods

Table 11.1 Endometrial receptivity scoring system (Zhang et al. 2022)

Parameter	Score 0	Score 1	Score 2
Endometrial thickness	<8 mm	8–14 mm	>14 mm
Endometrial volume	<3 mL	>3 mL	
Echo of the functional layer of endometrium	Heterogeneous	Homogeneous	
Endometrial central echogenic line	Absent	Present	
Endometrial peristalsis	Absent, positive, both positive and negative	Nondirectional	Negative
Endometrial blood flow	Absent, no more than 1/2	More than 1/2	Reaching the endometrial surface

are unsatisfactory, as neither the endometrial thickness nor the echogenic pattern correlates with the histological findings obtained from biopsy, though these criteria are scored and widely in use today as in Table 11.1. Basically, there is no known reliable method or technique to check whether the endometrium is synchronous with the time of embryo transfer (Horcajadas et al. 2007).

11.8 Gene Expression Profiling

Among many techniques such as genomics, epigenomics, proteomics, and transcriptomics, the latest has been adopted due to its accuracy and reproducibility in the endometrium. Transcriptomics is endowed with a molecular profile, signified as a transcriptome signature, which has also shown consistency with histological findings (Mahajan 2015). Microarray technology has shown good conduct with gene expression analysis studies as diagnostic tools. The Win (Window of Implantation) test is developed as a quantitative RT-PCR-based diagnostic tool to check the expression of specific marker genes.

Endometrial receptivity array (ERA) is a recent emerging tool based on microarray technology, developed and patented by *Igenomix*, to characterize gene expression for a set of 234 genes important for endometrial receptivity. The applicability of ERA lies in the transcriptional stratification of the endometrium as receptive, pre-receptive, or post-receptive. ERA is a clinical bioinformatic predictor designed to detect embryo-endometrial synchrony. ERA has emerged as a popular genetic tool with a specificity of 0.8857 and sensitivity of 0.99758 for endometrial dating and a specificity of 0.1571 and a sensitivity of 0.995 for pathological classification (Díaz-Gimeno et al. 2011). It functionally helps in understanding the endometrial-luteal phase differentiation. For infertile women who suffer from displaced WOI, the ERA test has emerged as a promising tool (Liu et al. 2022). With the insights gained in gynecological pathology, ERA offers a personalized approach to WOI identification and embryo transfer for use in IVF. It can be beneficial to patients with adenomyosis, endometriosis, and chronic stages of

endometritis. To extend the application of ERA, the transcriptome profile has been checked in all phases of the menstrual cycle, such as proliferative, early secretory, mid-secretory, and later-secretory phases, which shows promising and excellent features in diagnosis along with applicability in defining the chances for endometriosis and cancer (Habermann et al. 2011; Talbi et al. 2006). ERA is a reproducible and accurate tool that overcomes the limitation of inter-cycle variability. There have been suggestions that the transcriptional profile does not change over a short period of time (1–2 years), and, hence, one may not need to repeat it unless there are significant changes with respect to health, lifestyle, and metabolic profile.

Other significant oligonucleotide microarray-based studies were conducted on various platforms, such as the Human Genome U95A Array, Affymetrix GeneChip® Array (Riesewijk et al. 2003), and CodeLink UniSet Human I Bioarrays Slides (Amersham Biosciences, Piscataway, NJ) that give a global transcription profile for proliferative and secretory phases on an array that contains 10,000 gene probes belonging to well-annotated mRNA sequences. The CodeLink array is a 3D 30-mer oligonucleotides deposited gel matrix with high sensitivity that detects minimal differences in gene expression. It detects and quantifies subtle regulatory relationships among genes in the endometrium (Otsuka et al. 2007).

11.9 Metagenomics as an Emerging Tool in Endometrial Receptivity

The Endometrial Microbiome Metagenomic Analysis (EMMA) Test gives an estimate of the number of microorganisms in the uterine cavity and whether they are optimal or not. There is a comprehensive test called the EndomeTRIO test that gives results on the assessment of ERA, EMMA, and ALICE (Analysis of Infectious Chronic Endometritis) (Aghajanova et al. 2008). It is well accepted that endometrial microbiome analysis represents one of the most exciting and interesting acquisitions. It has the potential to aid clinicians in choosing the most useful markers for clinical practice. From research hitherto, transcriptomics would be a milestone in better understanding of genetic reasons for impairment in receptivity.

Maekawa et al. (2017) performed genome-wide mRNA expression analysis on thin endometrium at the mid-luteal phase, providing evidence that thin endometrium is associated with inflammation and oxidative stress that counts for infertility in women (Maekawa et al. 2017). This study showed 318 upregulated genes and 322 downregulated genes in the thin endometrium, compared to the control endometrium. Due to the fact that a microarray-based expression profile is constrained by the nature and specificity of the probes, gene profiling with the help of next-generation sequencing holds the potential for metagenomics. NGS is a precise and comprehensive tool to assess coverage and quantify global gene expression (Ben Rafael 2021). The noninvasive RNA-seq-based endometrial receptivity test (nirsERT) for transcriptome sequencing analysis of the uterine fluid combined with the random forest algorithm is an emerging potential diagnostic tool (He et al. 2021). It is the first evidence of transcriptomic profiling from uterine

Table 11.2 The list of commercial endometrial receptivity determining genetic tests

Sr. no.	Test name	Offered by	Detect number of genes	Reference
1.	WinTest quantitative RT-PCR	INSERM	11	Haouzi et al. (2009)
2.	ERPeak RT-PCR	CooperSurgical (USA)	40	Enciso et al. (2018)
3.	ERMap RT-PCR	IGLS (Spain)	40	Enciso et al. (2018)
4.	BeREADY Targeted Allele Counting by sequencing	Competence Centre on Health Technologies Ltd. (Estonia)	67	Altmäe et al. (2010)
5.	ERT	Yikon (China)	100	Not published in peer-reviewed journal
6.	BioER	Bioarray (Spain)	72	

fluid to show aberrant and differential gene expressions in human ER. The list of commercial endometrial transcriptomic tests is provided in Table 11.2.

11.10 Conclusions and Future Perspective

Far beyond morphological imaging-based assessments through sonography, the applicability of emerging next-generation sequencing tools to determine endometrial receptivity on genetic grounds has offered new reliable diagnostics. Based on the current availability of resources targeted at carefully assessing the endometrial receptivity before implantation in assisted reproductive technologies, it is crucial to dive deep into genomics for creating avenues for personalized healthcare strategies. The arrival of new panels targeting distinct crucial genes along with their differential regulation encompasses the genetic catalogue of the endometrium in real time. The transition of ERA test from microarray to NGS shows the rapidly evolving technology. The new tools utilize the epigenome and functional microRNAs for an in-depth understanding of cross talk and regulation in the endometrium, which is essential for receptivity and a successful pregnancy. As routine checkups do not evaluate fertility status in the female population, experimental validation is necessary to comprehend endometrial receptivity-based infertility in women. In order to identify promising biomarkers and molecular pathways involved in mid-secretory endometrial functions, an omics-based approach must be aimed at the diagnosis of inflammatory-based defects in endometrial receptivity. Omics tools such as genomics, transcriptomics, and epigenomics serve as potential diagnostic tools in determining inflammation in the endometrium and malfunction in endometrial receptivity. Recent studies have reported downregulation of HDAC3 in endometriosis, which is associated with impaired hormonal signaling and

receptivity, demanding deeper investigations into the epigenome for the identification of new genes/epigenetic markers. The identification of miRNA-mRNAs would reveal molecular pathways in women's infertility and generate new information on underlying transcriptomic mechanisms using RNA-seq. The art of precision medicine can blossom with the advent of NGS technologies and the identification of differential gene expression in ER. This could generate a cost-effective tool for personalized screening of female sex steroid hormones based on the assessment of endometrial receptivity and, hence, help in the optimization of treatment required at the individual level. Also, advanced microarray-based techniques and RNA transcriptome sequencing in biopsy samples and uterine fluid secretome can lay the foundation for better and non-invasive diagnosis and efficient treatment.

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Prenatal Genetic Testing

12

Fiona Olvitta D'Souza and Sarabpreet Singh

Abstract

Screening pregnancies for genetic disorders has become an integral part of antenatal care. Presently, a wide variety of prenatal screening tests in combination with ultrasound findings are offered in the first and second trimesters to evaluate the fetal risk for chromosomal anomalies. Chromosomes 13, 18, and 21 and sex chromosome aneuploidies are commonly evaluated based on their incidence and the possibility of live birth in fetuses carrying these aneuploidies. Prenatal screening tests can also be used to predict the risk of neural tube defects and preeclampsia. Due to the screening nature and limited sensitivity of biochemical testing, contingent testing with cell-free DNA-based noninvasive prenatal testing (NIPT) provides excellent detection of at-risk patients, with a sensitivity reaching up to 100% for trisomy 21. Standard guidelines, however, warrant care while interpreting reports of screening tests, and clinical decisions in high-risk cases are entirely based on invasive prenatal testing, which is the most accurate description of the genetic status of the fetus. Given the limitations of noninvasive prenatal screening tests, it is recommended that both noninvasive and invasive testing options be delivered to patients to help them make informed decisions. Invasive prenatal testing makes it possible to study a plethora of genetic conditions like aneuploidies, insertions, deletions, and mutations. Advancements in molecular diagnostic techniques has made it possible to deliver high-throughput data on a shorter timeline, allowing clinicians to make quick and informed decisions in the management of high-risk pregnancies.

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Keywords

Prenatal genetic testing, NIPS · Amniocentesis · Karyotyping · Microarray · Invasive prenatal testing · Reproductive medicine · Genetic disorders · Chromosomal aneuploidies

12.1 Introduction

Prenatal genetic testing provides information on the genetic health of the fetus in early pregnancy and plays a vital role in the decision-making and management of genetic disorders. Prenatal testing can reduce the burden of genetic disorders and congenital disabilities that cause significant postnatal disabilities and a reduction in the quality of life. Universal prenatal screening protocols are in place to address common genetic disorders and congenital abnormalities such as Down syndrome, Edwards syndrome, Patau syndrome, and neural tube defects. These tests are based on screening maternal blood, which enables the selection of patients who require an additional and detailed diagnosis. Prenatal genetic testing is most often preceded by genetic counseling due to the sensitive nature of knowing the genetic health of the fetus and to manage the stress endured by pregnant women during the anticipation of the results.

Prenatal genetic tests are broadly divided into prenatal screening and prenatal diagnostic tests. Prenatal screening tests are used to identify women at risk of carrying a fetus with a chromosomal abnormality. Screening tests are designed to focus on the most common trisomies and do not exclude the possibility of other genetic disorders. Screening tests merely provide risk prediction; they are reported as "high risk" or "low risk." While a high-risk screening report warrants further diagnostic testing, a low-risk report merely means that the patient has a low probability of carrying a child with the syndromes screened for. A low-risk report does not entirely eliminate the risk of having an affected child. Therefore, screening tests have to be interpreted carefully, along with the ultrasound report, bearing in mind the clinical history and ethnicity of the patient.

Prenatal genetic counseling must be offered to all pregnant women so that they understand the specific conditions that are included in such tests and to assess if additional genetic screening must be employed to identify the carrier status of the expecting parents.

12.2 Prenatal Screening Tests

Ultrasonography has been used for several years to periodically monitor pregnancy and is a noninvasive approach to evaluating the morphological appearance of the fetus in utero. However, the detection ability of ultrasound-based screening for genetic disorders is limited by expertise as well as the fact that most abnormalities may be missed due to poor visualization of the fetus. To overcome the limitations of

Table 12.1 Maternal serum screening test used for screening fetal chromosomal aneuploidies of chromosomes 13, 18, and 21

Screening test	Parameters	Detection rate	False-positive rate
Combined screening (dual marker)	PAPP-A + free βhCG + NT	85–90%	5%
First-trimester quadruple marker	PAPP-A + free βhCG + PlGF +AFP + NT	95%	5%
First-trimester Pentascreen	PAPP-A + free hCGß + AFP + PlGF + inhibin A	93%	5%
Integrated screening	NT and PAPP-A (first trimester), hCG, uE3, AFP, inhibin-A (second trimester)	85–90%	1–2%
Sequential screening	NT and PAPP-A (first trimester), hCG, uE3, AFP, inhibin-A (second trimester)	85–90%	1.5–2.5%
Second-trimester quadruple marker	Free βhCG +AFP + uE3 + inhibin -A	80–85%	5–8%

ultrasound-based screening, screening tests based on the combined analysis of maternal age, nuchal translucency (NT), and maternal blood for biochemical markers associated with the most common genetic syndromes were developed (Table 12.1). Any high-risk screening report was then followed by genetic screening of cell-free DNA.

12.2.1 Noninvasive Prenatal Screening (NIPS)

During the prenatal period, maternal blood contains cell-free DNA (cfDNA) from the mother as well as the fetus. It is therefore possible to assess the fetus for specific genetic disorders by noninvasive prenatal screening (NIPS) of maternal blood. Fetal cfDNA can be reliably detected in maternal blood by week 10 of gestation. Advances in next-generation sequencing and the development of new technologies in NIPS have now made it possible to not only detect chromosomal aneuploidies across all chromosomes but also identify fetuses at high risk of select microdeletions and inherited mutations by assessing cf DNA in maternal plasma. NIPS has high sensitivity (100, 100, and 100%) and specificity (99.89, 99.89, and 99.89%) for the detection of trisomies of chromosomes 21, 18, and 13, respectively (Chen et al. 2020). The cfDNA that is being tested in NIPS is of placental origin and not directly from fetal tissue; therefore, results from NIPS are to be interpreted as a screening tool. It is obligatory to confirm positive NIPS results by invasive prenatal sampling by chorionic villus sampling (CVS) or by amniocentesis (Dondorp et al. 2015; Srebniak et al. 2018). “False”-positive and “false”-negative results observed in NIPS can be attributed to the possibility of feto-placental mosaicism, which warrants confirmatory testing.

The cfDNA analyses are currently limited when compared to chromosomal microarray and exome sequencing in invasively obtained fetal samples, but the noninvasive character of this test led to a rapid acceptance of this analysis in the general population (the NIPS is now offered in all pregnancies). The genotype-first approach is therefore already widely applied in the prenatal screening setting.

Most commercially available NIPS tests are able to predict risk in fetal fractions above 4%. Gestational age below 10 weeks and maternal obesity are among the most common causes in cases where NIPS is inconclusive or unreportable, as the cell-free DNA content in such cases will fall below the required cutoff of 4% fetal fraction. False-positive results have also, on occasion, been associated with maternal malignancies.

In twin pregnancies, NIPT performance has been observed at a sensitivity of 100% and a specificity of 99.7% (Motevasselian et al. 2020), making it a reliable screening tool in twin gestations. However, in the case of multiple gestations greater than 2, it is not advisable to perform NIPS due to lower accuracy and the limitation of not knowing which of the fetuses could be at high risk. In cases of multiple gestations manifesting any anomaly on ultrasound, it is advisable to perform invasive prenatal testing and not NIPS (Fig. 12.1). NIPS is not recommended in cases of intrauterine demise as the etiology of fetal demise includes evaluation of nongenetic causes. In cases of vanishing twins or fetal reduction, it is recommended to wait a minimum of 4 weeks from the vanishing event to perform maternal serum screening and a minimum of 4–8 weeks to perform NIPS to avoid the risk of false positives.

12.2.2 Prenatal Diagnostic Testing: Invasive Prenatal Diagnosis

Prenatal diagnosis of genetic disorders can be achieved through invasive procedures with high accuracy. Screening tests for fetal aneuploidy are noninvasive, have no risk to the fetus, are affordable, can be applied to the majority of pregnant women, but do not allow a definitive diagnosis. Therefore, any high-risk result is mandated to be followed up and confirmed by an invasive prenatal diagnosis. The diagnosis of disorders prior to birth allows patients to make informed decisions about pregnancy and also allows the medical team to help develop treatment, intervention, and support for the expecting parents and the baby. Invasive prenatal diagnostic procedures such as chorionic villus sampling (CVS) and amniocentesis carry a minimal risk of 0.5% of a miscarriage (Akolekar et al. 2015), and appropriate counseling in regard to this risk is recommended.

12.2.2.1 Indications for Invasive Prenatal Testing

The most common indication for consideration of invasive prenatal testing is an increased risk of fetal chromosome abnormalities:

- Advanced maternal age (35 years old or older at the estimated date of delivery)
- A high-risk maternal serum screening
- Previous child with a chromosome abnormality

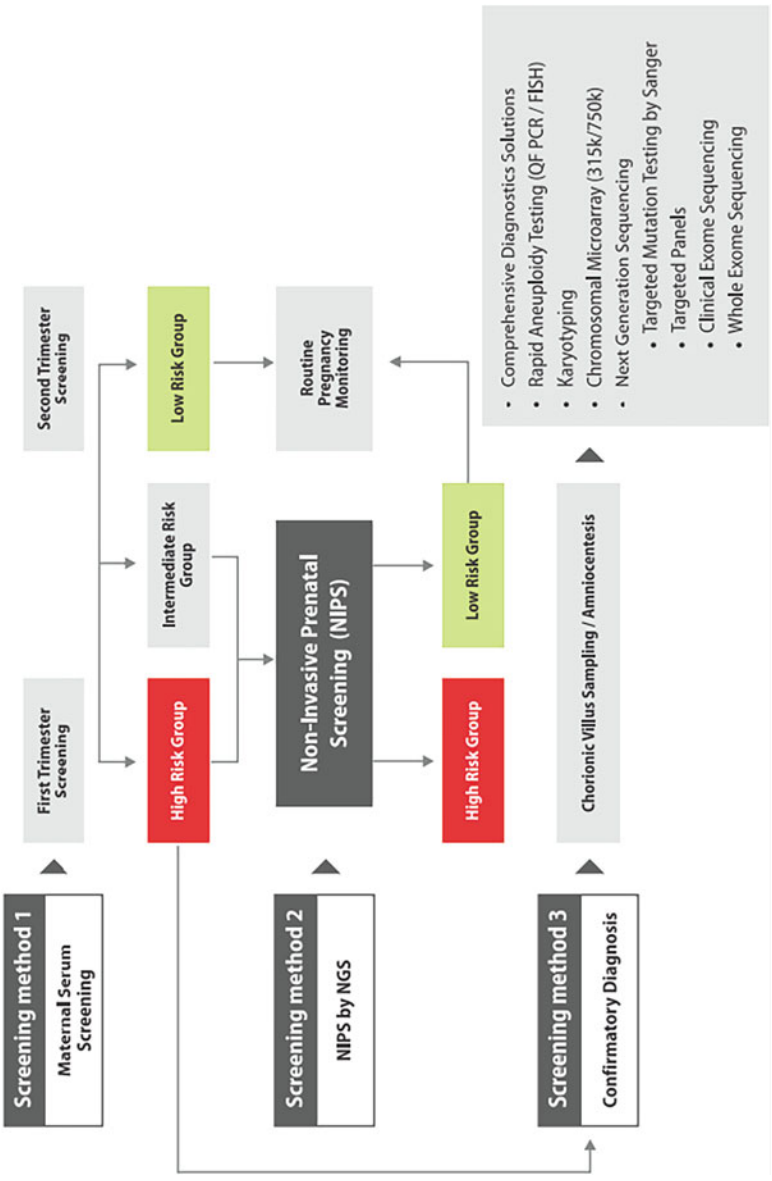


Fig. 12.1 Prenatal screening and diagnostic algorithm for fetal aneuploidies

- Parents being carriers for a chromosome rearrangement
- Increased risk of a detectable Mendelian disorder (earlier birth of a child or family member affected by a genetic disorder)
- Structural fetal anomalies detected by ultrasound
- Increased risk of multifactorial disorders, such as neural tube defects
- Detection of soft markers on ultrasound examination
- Exposure to teratogens (e.g., alcohol, chemotherapeutic agents, drugs)
- Increased risk of some perinatal infections

12.2.2.2 Chorionic Villus Sampling

Chorionic villus sampling (CVS) is performed between 10 and 13 weeks of gestation. During the procedure, a small tissue sample is obtained from the placenta by either inserting a thin needle through the woman's abdomen (transabdominal approach) or using a small catheter inserted through the cervix (transcervical approach). The method used depends on the location of the fetus and the placenta (Fig. 12.2).

The benefit of CVS over amniocentesis is that the fetal diagnosis is made earlier in pregnancy. This allows the patient to choose safer methods of termination if the results are abnormal and also helps resolve patient anxiety in cases where the results are normal (American College of Obstetricians and Gynecologists 2007). CVS is most commonly preferred in cases where an inheritable pathogenic variant has been identified in the parents.

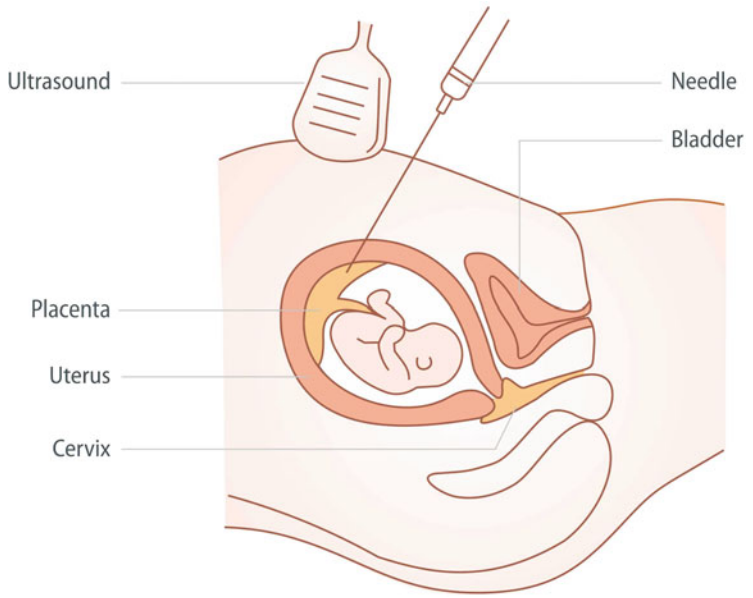


Fig. 12.2 Invasive prenatal sampling by chorionic villus sampling (CVS) can be performed between 10 and13 weeks of gestational age

12.2.2.3 Amniocentesis

Amniotic fluid is a clear liquid containing both free biochemical substances and the living cells of the fetus. Amniocentesis is performed after the 16th week of gestation. Extraction of amniotic fluid prior to 16 weeks of gestation has been shown to be associated with an increased risk of pregnancy loss and fewer amniocytes available for diagnostic testing. Amniocentesis involves the insertion of a 20- or 22-gauge needle into the amniotic sac under ultrasound guidance. Approximately 20–30 cc of amniotic fluid is obtained with this procedure (Fig. 12.3). It is preferred to sample all fetuses separately in cases of multiple gestations (twins and triplets) when an inherited condition is being assessed. In the case of fetal anomalies, only fetuses with anomalies may be sampled.

CVS or amniotic fluid can be subjected to cytogenetic studies (FISH or karyotyping) or molecular diagnostic techniques based on the indication. The results from the chromosomal studies by karyotyping usually take about 3 weeks due to the slow-growing nature of amniocytes in cultures, while rapid aneuploidy tests can provide quicker targeted information in as little as 48 h. There is a very small chance (approximately 0.5%) of pregnancy loss with amniocentesis. Leakage of amniotic fluid and slight bleeding can occur after amniocentesis. In most cases, both stop on their own.

12.2.2.4 Diagnostic Approaches in Invasive Prenatal Testing

The choice of diagnostic tests to be included in the post-invasive prenatal sampling of CVS or amniotic fluid is largely dependent on ultrasound findings. Procedures

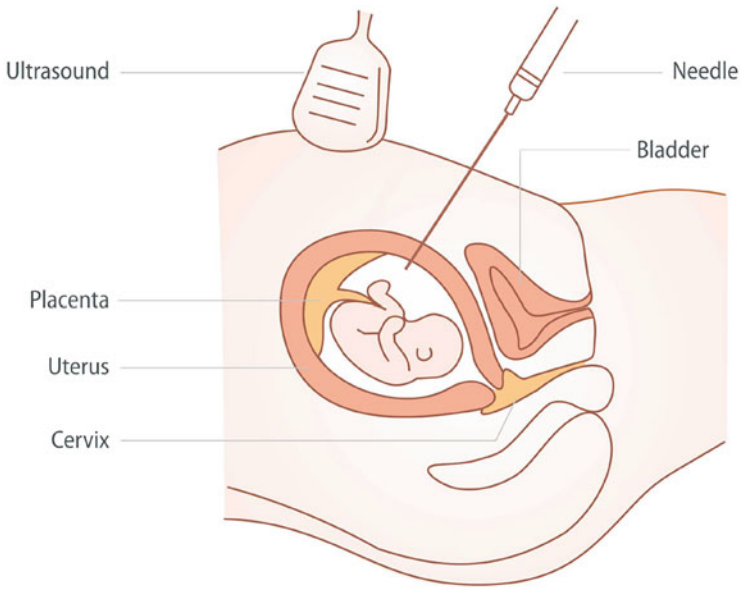


Fig. 12.3 Invasive prenatal sampling by amniocentesis after week 16 of gestation

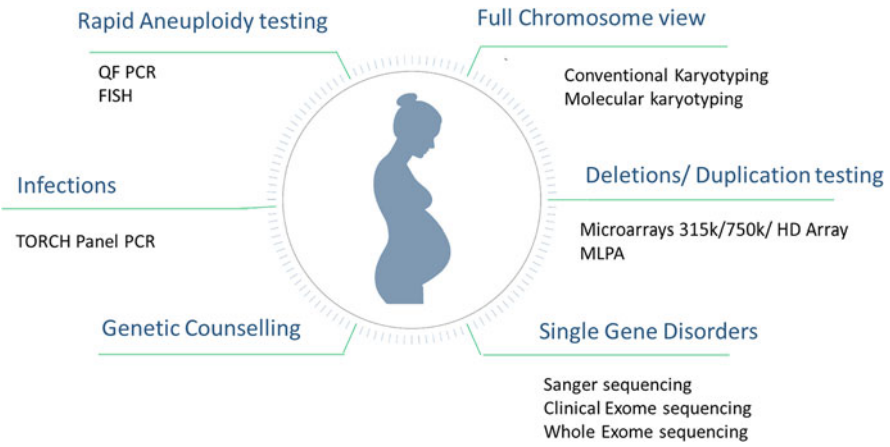


Fig. 12.4 Diagnostic techniques performed in prenatal samples

performed later than 16 weeks of gestation also allow the inclusion of early anomaly scans, which provide better phenotypic information and aid in the selection of more appropriate genetic tests. Rapid aneuploidy tests are employed to obtain quick results on aneuploidies in chromosomes 13, 18, and 21 and sex chromosome aneuploidies. While conventional karyotyping remains the gold standard technique to visualize the number and structure of chromosomes, advanced molecular diagnostic techniques like chromosomal microarrays offer higher-resolution testing to detect insertions and deletions across the genome. Targeted testing for specific deletions can also be performed by FISH or MLPA for known disorders. In conditions where specific inherited mutations are being assessed, it is essential that the mutation has been identified in the parents to facilitate a targeted approach to testing in CVS/amniotic fluid samples. In cases of advanced maternal age where a mutation is suspected either based on family history, previous affected fetuses, or ultrasound abnormalities in the present pregnancy, trio sequencing by panel of tests or exome sequencing by clinical exome or whole exome sequencing panels can be performed (Fig. 12.4) (Ghi et al. 2016).

12.2.2.4.1 Rapid Aneuploidy Testing

Rapid aneuploidy testing, such as quantitative fluorescence-polymerase chain reaction (QF-PCR) or fluorescent in situ hybridization (FISH), may be carried out CVS or amniotic fluid to test for specific chromosomes (21, 13, 18, X, Y). These tests provide results in 48 h and are commonly employed following a screen-positive result for maternal markers or in fetuses with ultrasound findings or ultrasound markers of common aneuploidies.

12.2.2.4.2 Fluorescent In Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) uses target-specific fluorescent probes that bind to the designated sites of the chromosome with sequence complementarity

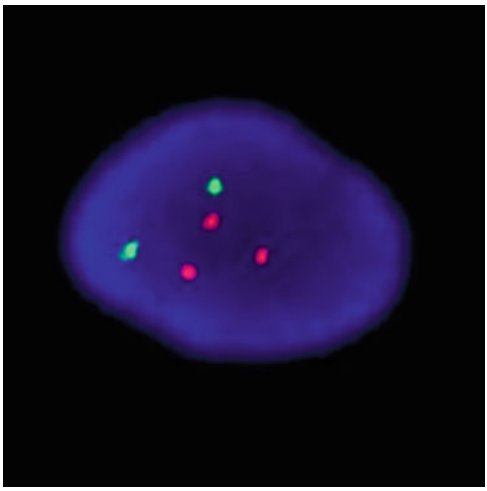


Fig. 12.5 Fluorescent in situ hybridization (FISH) analysis, amniotic fluid, uncultured cells representative of trisomy 21

(Fig. 12.5). The fluorescent probes are nucleic acids labelled with fluorescent groups that allow color labelling of different chromosomes. The high sensitivity (100%) and specificity (100%) of FISH toward the detection of trisomies in chromosomes 13, 18, 21, X, and Y and the speed with which the assays can be performed have made FISH a pivotal cytogenetic technique. FISH, however, is unable to detect structural chromosomal variants. Mosaic results on FISH often require conventional karyotyping to confirm the diagnosis. The limitation of FISH resides in the interference of signals in cases where amniotic fluid or CVS samples carry maternal blood contamination, leading to diffuse signals, and the fact that maternal cell contamination cannot be identified.

12.2.2.4.3 Quantitative Fluorescence-Polymerase Chain Reaction (QF-PCR)

Quantitative fluorescence-polymerase chain reaction (QF-PCR) is a laboratory technique used to amplify specific regions of DNA and quantify the amount of DNA present in those regions. QF-PCR can be used to detect the presence of additional chromosomes (aneuploidy) in patients. This can be useful for the detection of Down syndrome (three copies of chromosome 21), Edwards syndrome (three copies of chromosome 18), and Patau syndrome (three copies of chromosome 13). This technique can also be used to identify any sex chromosome aneuploidy. QF-PCR is a relatively less labor-intensive process when compared to FISH and allows quicker reporting. The advantage of QF-PCR over FISH resides in the fact that a minimum of 3–5 markers are assessed per chromosome and its ability to detect maternal cell contamination in prenatal samples. QF-PCR is limited in the detection of mosaicism below 15%.

12.2.2.4.4 Conventional Karyotyping

Karyotyping is a cytogenetic technique used to analyze all chromosomal structures and numbers in an individual. Karyotypes are prepared by culturing cells and

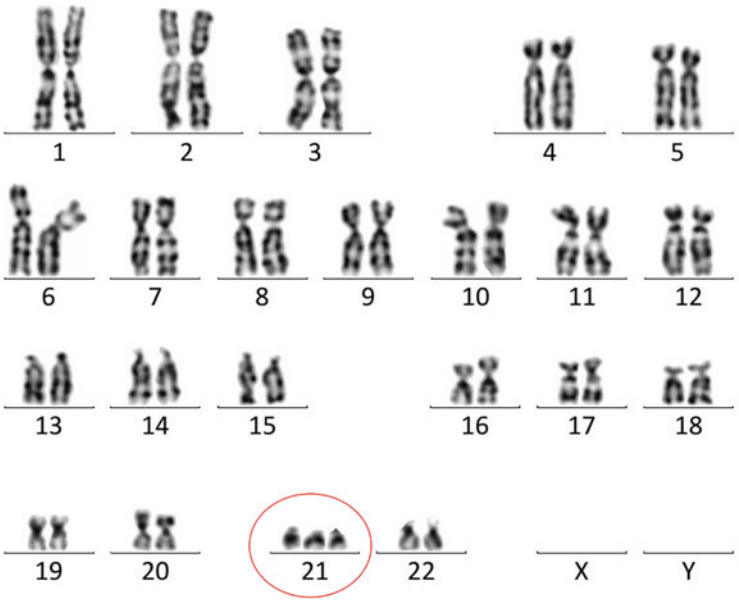


Fig. 12.6 Conventional karyotyping of amniotic fluid showing trisomy 21

artificially arresting them at metaphase, followed by visualization of chromosomes by G banding. G banding allows characterization of each chromosome by its unique banding pattern and length. Karyotypes help detect structural changes such as translocations and gross changes in chromosome numbers such as common whole chromosome aneuploidies like trisomies, monosomies, and triploidies. Karyotyping is known as the gold standard test for the structural analysis of chromosomes, as it is the only technique capable of detecting balanced translocations. Segmental aneuploidies and loss or gain of genomic material can be detected down to a resolution of 5 Mb with this technique (Down syndrome) (Fig. 12.6). Karyotyping is also a sensitive test to detect low levels of mosaicism.

12.2.2.4.5 Chromosomal Microarray

Chromosomal microarray (CMA) analysis is a high-resolution, genome-wide analysis technique used to identify chromosomal and sub-chromosomal gains and losses. It has an unprecedented advantage over standard cytogenetic tests like karyotyping and FISH as it has higher resolution and the ability to detect deletions and duplications down to 50–100 Kb (Callaway et al. 2013).

CMA has the advantage of detecting the following:

- Submicroscopic chromosomal deletions and duplications [copy number variations (CNV)]
- Regions of homozygosity (ROH)
- Regions exhibiting loss/absence of heterozygosity (LOH)

- Uniparental isodisomy (UDP)
- Low-level mosaicism

De novo insertions and deletions in the genome have been known to be strongly associated with most prenatal ultrasound anomalies. Therefore, CMA is considered a powerful tool for identifying the genetic etiology in prenatal testing. In fact, the American College of Obstetricians and Gynecologists (ACOG) and the Society for Maternal-Fetal Medicine (SMFM) recommend that chromosomal microarrays can be considered a replacement for the conventional karyotype in CVS and amniotic fluid samples in cases where structural anomalies have been identified by ultrasound (American College of Obstetricians and Gynecologists Committee on Genetics 2013; Hay et al. 2018).

The test looks for imbalances in the amount of chromosomal material between DNA from normal control and a patient's DNA (or DNA from the fetus present in the CVS or amniocentesis sample). Microarrays come in several resolutions and are selected based on genome coverage. It should be noted that the key limitation of CMA is that it cannot detect balanced translocations and mutations.

Maternal cell contamination (MCC) testing is an essential component of molecular diagnostic testing in prenatal samples. The presence of maternally derived cells associated with fetal sampling procedures such as chorionic villus, amniotic fluid, or the product of conception poses a significant risk of misdiagnosis. This is particularly of concern in prenatal samples, where a decision on continuing the pregnancy is based on genetic tests and the presence of maternal DNA can mask pathogenic insertions or deletions. Therefore, maternal cell contamination studies are performed to ensure the fetal sample is not contaminated with maternal DNA. MCC is performed by assessing a series of short tandem repeat (STR) markers. For this purpose, DNA extracted from the fetal sample and maternal blood undergoes multiple STR typing, in which different alleles present in 16 STR loci from chromosomes 2, 3, 4, 5, 8, 7, 11, 12, 13, 16, 18, 19, 21, X, and Y are amplified in a single reaction, followed by capillary electrophoresis. The fetal and maternal STR profiles are then studied for the presence of a second maternal allele in fetal DNA, as the fetal sample will have one maternal and one paternal allele. In cases where a prenatal sample is found to be contaminated with maternal DNA, a repeat sample may be requested by the diagnostic lab, depending on the percentage of MCC.

12.2.2.4.5.1 Recommendations for the Use of CMA

Most medical associations agree that CMA should be considered the test of choice in cases of significant structural anomalies, developmental delay, and intellectual disabilities in index cases with no prior family history of inherited disorders, autism spectrum disorder, or multiple congenital anomalies (Miller et al. 2010).

12.3 Next-Generation Sequencing (NGS)

Fetal abnormalities detected with ultrasound can range from subtle anomalies such as increased nuchal translucency or absent nasal bone to well-pronounced anomalies involving multiple organs such as hydrops fetalis, polycystic kidneys, skeletal malformations such as achondroplasia, etc. These conditions may be multifactorial or caused by environmental or genetic variations. Routinely applied molecular diagnostic tests have varying detection abilities for chromosomal abnormalities. Studies have reported that QF-PCR can enable rapid detection of autosomal trisomies in 30% of cases with USG anomalies. Karyotyping and CMA can detect chromosomal imbalances in up to 6.5% of structurally abnormal fetuses and have a resolution limit of 20 kb (Callaway et al. 2013). It is also necessary to acknowledge that in about 60% of pregnancies, a genetic cause often remains unidentified, and therefore decisions on the management of the pregnancy are often dependent on the severity of the anomaly. De novo or inherited pathogenic mutations also contribute to a significant proportion of prenatal anomalies. Exome sequencing panels can aid in the identification of mutations when recurring phenotypes are visualized antenatally. Exome sequencing also allows the detection of metabolic disorders that do not manifest as anomalies on ultrasound during the antenatal period (Lord et al. 2019).

The exome constitutes the protein-coding region of the genome. Exome sequencing by next-generation sequencing (NGS) evaluates the coding regions and splicing junctions of nuclear-encoded genes. About 85% of known disease-causing variants occur within the 1% of the genome containing the exome.

Exome sequencing can identify disease-causing DNA variants within the coding regions of the genome (exons) or flanking protein-coding regions (splice junctions). Clinical presentation plays a pivotal role in the interpretation of exome sequencing data. Exome sequencing has allowed identification of the underlying genetic etiology in about 25–30% of children with an undiagnosed genetic disorder. Several different exome sequencing panels are commercially available. When a specific phenotype has been identified, a gene-specific approach can be selected, while larger panels are opted for when multiple overlapping conditions may be suspected. For example, cases of ultrasound evidence of polycystic kidneys are strongly associated with autosomal recessive polycystic kidney disease, and a targeted approach with sequencing analysis of the *PKD1* and *PKD2* genes can be opted for. However, while selecting targeted approaches, differential diagnosis has to be factored in as prenatal testing is time sensitive. Therefore, in general practice, larger comprehensive panels, such as clinical exomes or whole exomes, can be chosen. In cases where an inherited mutation is suspected, particularly in cases of consanguineous marriages, it is essential that parental testing and mutation diagnosis be established prior to testing the fetus. The determination of the pathogenicity of a mutation will help in risk assessment and determine if invasive testing is necessary. In cases where parental testing has not been performed, whole exome sequencing by "trio analysis" is advisable where parental testing is performed concomitantly with the prenatal sample. This can avoid time-consuming segregation analysis, which becomes necessary to interpret the pathogenicity of novel variants. The limitation of larger

panels, however, is the possibility of identifying variants of unknown significance (VUS), which can further cause difficulties in the clinical decision-making process. Identification of the VUS can also cause a significant psychological burden to expectant parents; therefore, exome sequencing is recommended with appropriate genetic counselling addressing the possibilities of results following such tests.

12.4 Conclusion and Future Perspective

The information provided by genetic diagnostic testing plays a crucial role in decision-making and the management of cases with numerical and structural chromosomal anomalies detected through a prenatal ultrasound. The identification of fetal chromosomal defects has an important impact on the course of the pregnancy and the parents. An accurate characterization of the fetal chromosomal defects has implications for the phenotype-genotype correlation. Prenatal genetic testing helps couples decide regarding the continuation of the pregnancy or elective abortion and provides important information for future reproductive options in order to ensure the birth of a healthy baby.

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Genetic Testing in Recurrent Pregnancy Loss

13

Priyanka Verma

Abstract

Human pregnancy is a surprisingly inefficient process; approximately 5–10% of clinically recognized pregnancies end in miscarriages. Approximately 2–5% of RPL cases are due to a genetic abnormality in the fetus or the parents. RPL couples are often advised to visit reproductive experts to ascertain the cause of their repeated losses. Several techniques are used to detect the genetic defects, including karyotyping, fluorescence in situ hybridization (FISH), array comparative genomic hybridization (aCGH), single-nucleotide polymorphism (SNP) microarrays, and next-generation sequencing (NGS). The choice of technique varies depending on whether fetal tissue or the parents are being tested. This chapter focuses on the methods used for genetic testing in RPL cases. Limited and focused genetic testing is recommended by the European Society of Human Reproduction and Embryology (ESHRE) for RPL couples as a part of the diagnosis or for explanatory purposes.

Keywords

Recurrent pregnancy loss · Genetic testing · Genetics of pregnancy loss · Product of conception · Reproductive medicine · Infertility

13.1 Introduction

Recurrent pregnancy loss (RPL) is one of the most common public health issues worldwide, with an incidence rate of about 3% of all conceptions (Patki and Chauhan 2016). According to the American Society of Reproductive Medicine

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(ASRM) and the European Society of Human Reproduction and Embryology (ESHRE), RPL is defined as the loss of two or more consecutive miscarriages between 20 and 24 weeks of gestation, excluding molar and ectopic pregnancies (Christiansen et al. 2018; Practice Committee of the American Society for Reproductive Medicine 2012). Various factors contributing to RPL include environmental, genetic, anatomical, endocrine, and inflammatory agents (Sultana et al. 2020; Tur-Torres et al. 2017). Additionally, several pieces of evidence have shown a high rate of malnutrition, advanced maternal and paternal age, over- or underweight mothers, smoking, and alcohol consumption to be linked with the risk of RPL. Based on the aforesaid etiological factors, risk factors for RPL fall into three broad categories: (1) genetic defects (e.g., chromosomal abnormalities or gene mutations), (2) alterations on the maternal side (e.g., immunological, genetic, immunogenetic, anatomical, and endocrine factors) and lifestyle, (3) others (e.g., environmental pollutants). Recent studies suggest that paternal factors are also associated with RPL, which are usually ignored during diagnosis. Paternal factors that may contribute to pregnancy loss include sperm DNA fragmentation, oxidative stress, histones and their posttranslational products retention at specific genomic sites, short telomeres, and epigenetic changes in sperm (Kumar et al. 2016; Peuranpää et al. 2022). Paternal factors are also responsible for the majority of sex chromosome trisomies, i.e., 47, XXY, and 47, XYY, which contribute to pregnancy loss due to abnormal fetal development. Sperm from couples with a history of RPL were found to display an increase in sex chromosome trisomy compared with control samples. Oligoasthenoteratozoospermia (low number of sperm, poor sperm movement, abnormal sperm shape) patients have the highest rate of sex chromosome disomy as well as high rates of disomy of chromosomes 18 and 21 when compared to controls (Rubio et al. 2001). Male-driven disomies are more common with advanced paternal age. Although the etiology of RPL is varied and controversial, numerous studies suggest that it is a multifactorial process.

About 50% of RPL cases are caused by genetic defects, which can be divided into chromosomal and monogenic disorders (van den Berg et al. 2012). Chromosomal abnormalities are one of the causes of RPL, and they could be numerical (aneuploidies) or structural (deletions, inversions, and translocations) (Page and Silver 2016). Chromosomal abnormalities were found very frequently in the product of conception (POC) (Romero et al. 2015). The ASRM and ESHRE are the two societies that suggest overall guidelines to clinicians with the finest available evidence for the treatment and management of women with RPL. In 2012, the ASRM recommended anatomic uterine factors screening, antiphospholipid syndrome, hormonal disorders, and parental karyotyping. New guidelines from the ESHRE, 2017, omitted POC and parental genetic analysis from routine screening, except for RPL couples willing to undergo individual risk assessment, including a prior family history. ESHRE mentioned that the genetic analysis of POC is not routinely recommended, but it can be used to provide some explanation to the patients. The array-CGH technique is recommended for POC testing because regular karyotyping/FISH cannot rule out maternal cell contamination (Christiansen et al. 2018; Practice Committee of the American Society for Reproductive Medicine 2012).

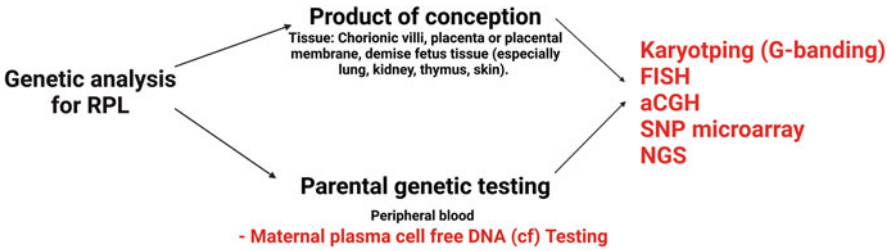


Fig. 13.1 Genetic testing techniques recommend by the ESHRE and ASHRM for RPL couples

In this chapter, we focus on the need for and methods of genetic testing in RPL. There are broadly two ways of undertaking genetic screening based on the sample available: (1) genetic analysis of the product of conception and (2) parental genetic analysis (Fig. 13.1).

13.2 Genetic Analysis of the Product of Conception (POC)

Fetal aneuploidy is considered to be a major risk factor for RPL. Fetal aneuploidy includes the loss or gain of extra chromosomes in the fetal tissue, which leads to a nonviable pregnancy or the offspring may not survive or may develop genetic defects (Driscoll et al. 2009). Increasing maternal age is believed to be a risk factor for fetal aneuploidy. All pregnant women with a history of RPL should offer fetal aneuploidy testing in the first trimester. Common first trimester fetal aneuploidies are trisomies 21, 18, and 13, as well as sex chromosome aneuploidies. The most probable reason behind these aneuploidies is meiotic nondisjunction during gametogenesis (LeFevre and Sundermeyer 2020). Another known genetic cause is balanced reciprocal, or a Robertsonian translocation, which usually occurs in 2–5% of RPL cases. To detect these aneuploidies, different types of genetic techniques are offered to the patients, which include fetal tissue karyotyping, comparative genomic hybridization (CGH), etc.

The traditional karyotyping method (G-banding) is one of the methods for determining chromosomal aberrations in the POC (Papas and Kutteh 2021). POC samples include chorionic villi, placenta or placental membrane, and demise fetus tissue (especially the lung, kidney, thymus, and skin). G-banding analysis can detect aneuploidy (missing or extra chromosomes), ploidy (extra sets of chromosomes), large deletions or duplications of chromosomes, chromosomal rearrangements, and chromosomal mosaicism (varying chromosome complements in different cells) as shown in Fig. 13.2.

Traditional karyotyping has several limitations for POC genetic analysis, such as a low live cell count, high chances of maternal cell contamination, labor-intensiveness, a high rate of culture failure, fetal mosaicism, and most of the time, the loss of POC tissue during miscarriage. Due to these limitations, the ESHRE

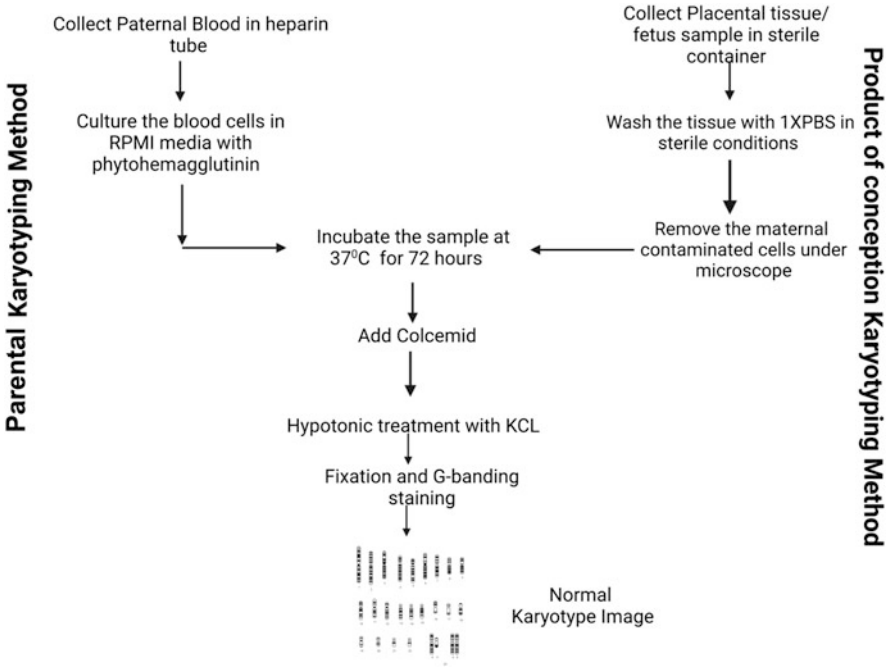

Parental Karyotyping Method
Collect Paternal Blood in heparin tube
Culture the blood cells in RPMI media with phytohemagglutinin
Collect Placental tissue/ fetus sample in sterile container
Wash the tissue with 1XPBS in sterile conditions
Remove the maternal contaminated cells under microscope
Product of conception Karyotyping Method
Incubate the sample at 37°C for 72 hours
Add Colcemid
Hypotonic treatment with KCL
Fixation and G-banding staining
Normal Karyotype Image

Fig. 13.2 A pictorial representation of parental and the product of conception traditional karyotyping techniques

recommended that POC genetic analysis be used for explanatory purposes (Marren 2018).

Many recent technologies can also be used for POC analysis, such as fluorescence in situ hybridization (FISH), array comparative genomic hybridization (aCGH), single-nucleotide polymorphism (SNP) microarrays, and next-generation sequencing (NGS). The advantages and disadvantages of the available techniques are described in Table 13.1.

13.2.1 Fluorescence In Situ Hybridization (FISH)

FISH is a fast, inexpensive tool for detecting fetal/paternal aneuploidy in RPL cases. The genetic diagnosis of the POC is usually ignored for financial and technical reasons. The FISH technique is a sufficient solution to all these problems because of its cost-effectiveness and flexibility in using sterile or fresh fetal tissue. The method is rapid, requires a small amount of fetal tissue, and saves the complexity of cytogenetic metaphase karyotype analysis (Fejgin et al. 2005).

FISH requires two basic elements: a DNA probe and target sequences. DNA probes are short sequences of single-stranded DNA that match our gene of interest. DNA probes are labeled with fluorophore nucleotides via direct (probes labeled with

Table 13.1 Advantages and disadvantages of genetic analysis techniques available for RPL

Screening Test	Specimen type	Advantages	Disadvantages
Parental karyotype	Blood of both parents	• Identifies balanced translocation carriers	• Small deletions cannot be detected
POC karyotype	Placenta, fetal tissue, Cord blood	• Identifies POC aneuploidy, balanced translocation	• Small deletions cannot be detected. • Labor intensive • Requires culture of live cells • High rate of culture failure. • High rate of maternal cell contamination
FISH	DNA samples extracted directly from the fetal tissue	• Rapid • Require small amount of fetal tissue and saves the complexity of cytogenetic metaphase karyotype analysis	• Small deletions cannot be detected • Base pair mutations cannot be detected
POC CMA	DNA samples extracted directly from the fetal tissue	• Imbalanced genomic alterations (microdeletions and microduplications) can be detected. • Can detect the absence of heterozygosity (AOH) and uniparental isodisomy (UPD) • Simultaneously and rapidly evaluates thousands of loci for copy number variations • Further characterizes chromosomal imbalances detected by karyotyping (e.g., marker chromosomes) • Detect polyploidies such as complete mole or partial mole pregnancies • Does not require cell culture	• Cannot detect balanced translocations • Base pair mutations cannot be detected. • Gains or losses in genome are not covered by the array • Low level mosaicism (< 20%) cannot be detected. • No link to the future pregnancy diagnosis
NGS	DNA/RNA samples extracted directly from parental blood	• Does not require cell culture • Identifies cryptic balanced chromosome rearrangements and copy number variants (CNVs)	• Expensive • Not generally covered by insurance
Maternal plasma cell free DNA	Cell free fetal derived DNA released from fetus cells in maternal plasma	• Detect fetal aneuploidies within the first and second trimesters • Early detection of major defects and prediction of pregnancy complications	• Expensive • Not generally covered by insurance

nucleotides contain a fluorophore) and indirect (modified nucleotides contain a hapten at which probes are labeled) labeling. Hybridization of the denatured labeled probe and the target sequences is allowed, followed by the analysis of the signal using a fluorescent microscope (Ratan et al. 2017). Common probes for chromosomes such as 13, 16, 18, 21, X, and Y are used for the detection of specific chromosomal aberrations in POC (Fejgin et al. 2005) and aneuploidy in sperm samples (Fakhrabadi et al. 2020) of RPL couples.

13.2.2 Array Comparative Genomic Hybridization (aCGH)

The CGH technique is gaining more interest for cytogenetic analysis of the POC. The CGH procedure is a little different from the FISH protocol. In CGH, the reference control DNA from healthy cells and the DNA extracted from the POC samples are labeled with fluorescent molecules having different fluorophores and subsequently hybridized in an amount equal to a set of normal metaphase chromosomes. Hybridized fluorescence is analyzed via computer software to identify the area of imbalance between the test and control samples. The areas of imbalance are known as copy number variations (CNVs) (Youssef et al. 2019).

13.2.3 NGS Approach

The next-generation sequencing (NGS) approach has transformed the postnatal diagnosis of genetic diseases. Nowadays, the NGS technique is very helpful in diagnosing the genetic etiology of RPL and miscarriages and provides a great deal of relief to patients and clinicians. It includes whole exome sequencing (WES) to look for pathogenic sequence changes in the coding genome or the candidate genes considered important for early embryonic development and pregnancy maintenance in cases where the single gene approach fails to identify the likely pathogenic mutation. Whole genome sequencing (WGS) can be used for the identification of novel chromosomal rearrangements and copy number variation (CNVs) in couples with RPL (Rajcan-Separovic 2020).

Al Qahtani et al. (2021)) studied a family trio (the dead fetus and the couple) using WGS and identified a novel homozygous exonic variation (NM_017419.3: c.680G > T) in the ASIC5 (acid-sensing ion channel subunit family member 5) gene in the dead fetus, whereas the couple was heterozygous for this mutation (Al Qahtani et al. 2021). Xiang et al. (2021) performed WES in a Chinese cohort and identified four strong candidate genes: FOXA2, FGA, F13A1, and KHDC3L for RPL (Xiang et al. 2021). They further validated that the knockdown of FOXA2 results in reduced expression of LIF (an important gene involved in implantation), thereby increasing the chances of pregnancy loss.

13.2.4 Maternal Plasma Cell Free Fetal (Cff) DNA Testing

Maternal plasma cffDNA testing is a new noninvasive technique that can detect chromosomal anomalies in the fetus with a specificity of 90%, a sensitivity of 82%, and an overall accuracy of 85%. CffDNA is found in the maternal blood and released by the placental cells (cytotrophoblast and syncytotrophoblast) during pregnancy. Usually, cffDNA testing is recommended after 8 weeks of pregnancy. CffDNA testing can detect chromosomal numerical abnormalities (aneuploidy) and extra chromosomes in the fetus and is relatively cheaper than other methods of genetic testing in RPL (Yaron et al. 2020). CffDNA testing can also detect mild-to-severe hemolytic diseases of the fetus (O'Brien et al. 2020). In this technique, DNA fragments of approximately 150–200 base pair (bp) size are isolated from maternal blood plasma. Excess tags of particular sequences suggest aneuploidy of that region or chromosome (Taglauer et al. 2014).

13.3 Parental Genetic Analysis

13.3.1 Peripheral Blood Karyotyping

The ASRM recommended peripheral blood karyotyping of parents to diagnose the cause of pregnancy loss, but the ESHRE suggested its use for conditional diagnosis. It can detect parental numeric and structural cytogenetic abnormalities, followed by genetic counseling (Papas and Kutteh 2021). Genetic counselors could discuss the results, i.e., both the specific chromosomes affected and the influence of chromosomal translocation on future conception and pregnancy. Any known structural abnormality or single gene mutation detected in RPL patients could be used for preimplantation genetic testing for future conception. Several reports have suggested that the majority of losses occur in the carriers of reciprocal translocations and inversions compared to Robertsonian translocations (Hyde and Schust 2015).

13.3.2 Single Gene Defects

Single gene defects have also been associated with RPL. Single gene defects include musculoskeletal gene mutations, hemoglobinopathies, immunological gene defects, metabolic and hormonal gene defects, X-linked genes, apoptosis related genes, angiogenesis and vasculogenic gene defects, etc. (Akdemir et al. 2020; Michita et al. 2019; Moghbeli 2019). Specific single gene defects associated with RPL have been listed in Table 13.2. High-throughput techniques such as whole exome and genome sequencing approaches are capable of detecting single gene defects in RPL patients. The identification of single gene defects in clinical practice may not be helpful for RPL patients because of their multifactorial nature and the complex genetics behind them. Integrated assessment of potential gene defects in the trio

Table 13.2 Summarized list of pathway-specific single gene defects

Pathways Name	Associated gene name	References
X linked disorder	MECP2, SXCI, AR	Sui et al. (2022)
Apoptosis-related genes	FAS, FAS-L, BAX, BCL, Annexin A5	Michita et al. (2019)
Musculoskeletal gene defects	PIEZO1, POMT1, FRAS1, COL1A1, DIS3l2	Najafi et al. (2021)
Immunologic gene defects	HLA-G, HLA-G1, HLA-G5, HLA-E, IL1B, IL6, IL10, IFNγ, TNFα, IDO2, TLR3, CR1, TRAF3IP1	Moghbeli (2019), Quintero-Ronderos et al. (2017) and Wysocka et al. (2021)
Thrombophilic gene defects	PAI-1, HPA-1, ACE, APOE, MTHFR, CD46, AR, LEPTIN, THBD	Moghbeli (2019) and Nair et al. (2012)
Mutations in specific enzymes	MTHFR, AMN, ALPP	Moghbeli (2019)
Angiogenic and vasculogenic gene defects	VEGF, miR-21, PTEN, NOS, P53, SULFI, ND1, FLT-1, EPAS-1	Moghbeli (2019)
Placental gene defects	MTRR, NUP98, CTNNA3	Moghbeli (2019)
Extracellular matrix protein	MMP10, MMP9, COL6A3, ADAMTS1, TNC	Quintero-Ronderos et al. (2017) and Wysocka et al. (2021)

analysis format (mother, father, and conceptus) will be helpful in understanding the causes of RPL and offering counseling or treatment (Hyde and Schust 2015).

13.4 Genetic Testing as a Diagnostic Tool and Therapy in RPL

Clinical and physical evaluation, including the family history of patients, is an essential factor in the diagnosis of the disorder and important for the selection and interpretation of genetic test results. Genetic testing is helpful for clinicians to diagnose the genetic causes of new RPL patients. The identification of a genetic cause is effective in helping RPL patients plan a pregnancy and beware of their birth outcomes. Although traditional methods, such as karyotyping, are cost-effective, they are unable to detect point and novel mutations. Non-culture-based techniques, such as aCGH and maternal plasma cff DNA testing, are more reliable and precise to overcome the challenges of the traditional methods (Hyde and Schust 2015; Sierra and Stephenson 2006).

Recent evidence suggests that preimplantation genetic screening (PGS) could be used as a therapy for RPL couples (Munné et al. 2005). Pregnancy outcome is improved by using PGS for aneuploidy loss and translocation carriers in RPL patients (Otani et al. 2006; Vitez et al. 2019). PGS facilitates the selection and transfer of euploid embryos in RPL patients, reducing the risk of clinical miscarriage and ongoing aneuploid gestations. RPL couples with an abnormal karyotype can try in vitro fertilization with preimplantation screening (PGS) in pursuit of a viable

pregnancy. The in vitro fertilization process involves oocyte pickup, selection of sperm from a healthy donor, embryo culture and biopsy, genetic diagnosis, and finally, the selective transfer of "unaffected" embryos (Allahbadia et al. 2020). This procedure lessens the possibility of passing certain chromosomal defects or monogenic diseases to the offspring when one or both parents are carriers. The other intention is to improve the IVF success rate. IVF with PGS is an invasive, time-consuming, and expensive process, but may be suitable in RPL cases (Griffin and Ogur 2018). However, a deeper understanding of the genetic causes of RPL will facilitate the use of PGS with IVF to overcome pregnancy loss in susceptible couples.

13.5 Conclusions and Future Perspective

Genetic testing in RPL cases can play a crucial role in explaining the failure and treating it. Genetic testing at the time of pregnancy loss should be done on the fetal tissue using techniques such as karyotyping or FISH, to identify chromosomal numerical or structural abnormalities or FISH to identify common chromosomal alterations, such as 13, 16, 18, and 21 trisomies and X and Y aneuploidies. This should be followed by array CGH or next-generation sequencing to identify copy number variations or single gene mutations contributing to RPL. In cases of pregnancy loss, chromosomal analysis should be undertaken to explain the loss and for future reference.

Cell-free DNA testing should be done in pregnant women to identify fetal chromosome aneuploidies, which can contribute to RPL. This can be done in a minimally invasive way using the mother's peripheral blood sample. If genetic testing at the time of pregnancy loss is not undertaken, the couple should undergo genetic testing to identify the probable genetic cause of RPL. This usually involves peripheral blood karyotyping of the couple, followed by genetic analysis to identify single gene defects so that the risk of future pregnancy loss can be calculated. Fortunately, some of the genetic alterations can be biochemically managed by supplementing certain vitamins and cofactors.

In cases of RPL associated with a genetic defect, in vitro fertilization can be used in conjunction with preimplantation genetic testing to minimize the risk of pregnancy loss. This reduces the burden of undergoing the emotional and physical trauma of pregnancy loss. In the future, there will be genetic screening panels for prospective parents and specific tests for RPL cases to genetically test their susceptibility and offer rational preventive measures.

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Genetic Testing in Pregnancy to Assess the Risk of Preterm Birth

14

Renu Bala and Rajender Singh

Abstract

Preterm birth (PTB) is one of the leading causes of infant and child mortality under 5 years of age. PTB infants have long-term health consequences and usually suffer from sepsis, respiratory distress syndrome, feeding difficulties, and the risk of adult metabolic diseases. Various studies have highlighted the role of a complex interplay between genetic (maternal and fetal) and environmental factors in controlling birth timing in humans. However, the molecular mechanisms involved in the pathophysiology of PTB are still poorly understood. In recent years, studies have suggested a significant contribution of genetic variations to the etiology of PTB. The obstetric/familial history of women with PTB and twin studies also emphasizes the role of genetic factors. In this chapter, we illustrate the plausible genetic factors associated with PTB risk and their suitability to assessing the risk of PTB.

Keywords

Genetic variations · Single-nucleotide polymorphisms · Preterm birth · Infertility · Genetic testing · Reproductive medicine · Premature birth · Preterm delivery

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14.1 Introduction

Pregnancy is a complex physiological phenomenon, the success of which is governed by a multitude of factors, such as the environment, genetics, and maternal–fetal factors (Goldenberg et al. 2008). The initiation of labor and parturition at term is regulated by precise coordination of several physiological pathways (maternal and fetal), culminating in cervical ripening, uterine contractions, and expulsion of the fetus (Smith 2007). A baby born alive before the completion of gestation or term (≥37 weeks) is called a preterm birth (PTB). PTB is either medically indicated or is a spontaneous event (due to premature labor or preterm premature rupture of membranes) (Goldenberg et al. 2008). PTB is among the leading causes of infant mortality and morbidity worldwide (Di Renzo et al. 2018). The etiology of PTB is multifactorial, and its incidence varies across countries, ranging from 5 to 18% (Harrison and Goldenberg 2016). Substantial research has been done to identify the risk factors for PTB (such as inflammation/intrauterine infections, uterine anomalies, obstetric history, and ethnicity), but the molecular mechanisms involved in triggering these pathways are still poorly understood (Goldenberg et al. 2008; Harrison and Goldenberg 2016) (Fig. 14.1). Some studies have also advocated the use of identifiable markers in these pathways as predictors of the PTB risk; for example, tumor necrosis factor-α (*TNF-α)* in maternal serum, fetal fibronectin and *IL1-β* in the cervical fluid, and *MMP-8* in the amniotic fluid (Honest et al. 2002; Jelliffe-Pawlowski et al. 2015; Kim et al. 2016). However, the sensitivity,

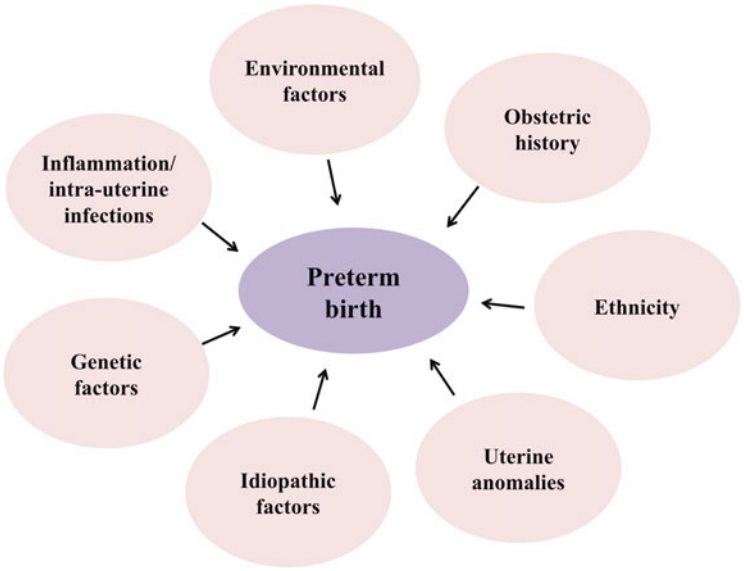


Fig. 14.1 Risk factors associated with preterm birth

specificity, and levels of such markers can vary across different trimesters as well as among individuals, making it difficult to employ them timely with successful intervention as well as to obtain reproducible results (Oskovi Kaplan and Ozgu-Erdinc 2018). Contrary to this, some researchers have suggested the use of genetic markers as an alternative advanced strategy to establish biomarkers for early diagnosis of PTB, as they are more accurate and stable across time and within individuals (Hee 2011). Various studies have suggested that genetic predisposition can increase the risk of PTB significantly (Menon et al. 2006; Plunkett and Muglia 2008; Sheikh et al. 2016). These studies have shown the association of genetic factors such as single-nucleotide polymorphisms (SNPs), copy number variations, differential methylation, translocations, inversions, and genomic imbalances as the main factors. Twin studies and cases with obstetric or familial history have further strengthened the role of genetic factors in PTB (Chen et al. 1996; Kazemier et al. 2014; Sheikh et al. 2016). In this chapter, we intend to review the genetic factors associated with PTB risk with the aim of identifying the potential genetic markers of PTB risk.

14.2 Familial and Obstetric Histories Suggest Genetic Risk Factors

Women with short interpregnancy intervals are known to have a higher risk of PTB. Interpregnancy duration shorter than 6 and 18 months increases the chances of PTB by 1.4 times (95% CI: 1.0–1.8) and 1.6 times (95% CI: 1.2–2.1), respectively (Kozuki et al. 2013; Männistö et al. 2017). PTB during a previous pregnancy is the strongest predictor of PTB in the current pregnancy, with about 30–40% increased risk (Kliegman et al. 1990). Studies have shown that if a woman had PTB during a previous pregnancy or if she/her sister was born preterm, then her risk of having a PTB increases by 4–6 (odds ratio) and 1.94–2.25 times (relative risk), respectively (Di Renzo et al. 2011; Ferrero et al. 2016; Koire et al. 2021). However, this risk is unaffected by the occurrence of PTB in a woman's paternal side family or her partner's side family, suggesting the role of genetic risk being passed from the female's lineage (Boyd et al. 2009). The presence of multiple fetuses and the birth timing during the current or previous pregnancy also affect the risk of PTB. The risk of preterm birth is increased by 57% (95% CI: 51.9–61.9%) if a woman with twin pregnancy had a previous preterm singleton pregnancy and by 25% (95% CI: 24.3–26.5%) after a previous singleton term pregnancy (Kazemier et al. 2014). On the other hand, a woman pregnant with a singleton fetus after a previous preterm twin pregnancy has a 10% (95% CI: 8.2–12.3%) reoccurrence risk and only 1.3% (95% CI: 0.8–2.2) risk of PTB if she had a previous twin term pregnancy (Kazemier et al. 2014).

14.3 Genetics of PTB Risk

14.3.1 Racial/Ethnic Divergence and Genetic Predisposition

Differences in the rates of preterm birth among women of different races and ethnicities also provide evidence for genetic predisposition to PTB risk. It has been shown that non-Hispanic black women have a higher risk of PTB as compared to Hispanic and non-Hispanic white women (Bediako et al. 2015; DeSisto et al. 2018; Martin and CDC 2011). In the United States, non-Hispanic black women have a 48% higher risk of PTB as compared to women of other racial groups (Rubens et al. 2014). The mechanism behind these racial disparities is not fully understood; however, the role of underlying genetic variations along with gene–environment interactions has been suggested (Rubens et al. 2014). The strong correlation of the *TNF-2* allele (present at −308 position) of the tumor necrosis factor-alpha (*TNF-α)* gene with PTB in African-American women (OR: 2.5, 95% CI: 1.4–4.5) as compared to Caucasians (OR: 1.6, 95% CI: 0.5–5.2) has been reported, and the presence of bacterial vaginosis further increases this risk (Macones et al. 2004). Similarly, a single-nucleotide polymorphism (SNP) (−656 C/T) in the *SERPINH1* gene (role in collagen biosynthesis) is associated with PTB risk, and it is more frequent in African-Americans (12.4%) as compared to European-Americans (4.1%) (H. Wang et al. 2006). It has also been shown that black women are at a higher risk of PTB, irrespective of their socioeconomic conditions (Kistka et al. 2007; Phillips et al. 2013), but counterevidence is also available (Braveman et al. 2015; Cooper et al. 2016). The role of obstetric history in pregnancy outcomes is also explained in the upper-middle-class Chinese population (Taiwan), where the main risk for PTB was prior abortions, PTB, and multiple gestations (Chen et al. 1996). A systematic review and meta-analysis showed no association of Asian and Hispanic ethnicity with PTB risk (Schaaf et al. 2013). A recent study has shown that non-U.S.-born Vietnamese, Chinese, Japanese, and Indian women have a lower risk of PTB as compared to U.S.-born Asian subgroups (Girsen et al. 2020), indicating the role of biological, societal, and environmental factors in contributing to PTB.

14.3.2 Role of Genetic Variations in PTB Risk

For the last two decades, the candidate gene approach has been utilized to identify genes involved in PTB. Single-nucleotide polymorphisms (SNPs) or other genetic variations (insertions, deletions, differences in repeat sequence of a few or more bases) in functionally relevant genes of crucial biological pathways (such as the inflammatory and tissue remodeling pathways) have been identified to be associated with adverse birth outcomes such as PTB (Hong et al. 2021; Menon et al. 2006; Nesin 2007; Plunkett and Muglia 2008; Sheikh et al. 2016; Wang et al. 2006). The majority of these SNPs are functional substitutions that lead to differential gene expression, resulting in altered end products. The SNPs in genes of the inflammatory pathway (*IL1-β, TNF-α, IL-6, IL-1ra, IL-4, IL-10*), toll-like receptor-4 (*TLR-4*),

matrix-metalloproteinases (*MMP-1, MMP-8, MMP-9*), vascular endothelial growth factor (*VEGF*), beta2-adrenergic receptor (*beta2-AR*), folate metabolism genes (*MTHFR, CBS, MTRR*), and other significant pathways involved in pregnancy maintenance (in mothers or neonates) affect the risk of PTB (Nesin 2007; Plunkett and Muglia 2008; Sheikh et al. 2016). However, this association is not uniform and varies among different populations and races (Nesin 2007). Differential risk among populations can be explained by gene–environment interactions, where a gene mutation increases the risk in the presence of certain environmental stimuli (such as bacterial vaginosis and vaginal-urinary tract infection) (Elias et al. 2021; Macones et al. 2004). The coexistence of two or more genetic variations and an unfavorable intrauterine microenvironment can further heighten the risk of PTB (Menon et al. 2006; Wang et al. 2000).

Contrary to the traditional approach of analyzing candidate genes, a more comprehensive approach known as genome-wide association study (GWAS) has also been employed to achieve a better understanding of genetic contributions to PTB risk. A genome-wide association study in 43,568 women of European ancestry found that variants in the *EBF1* (rs2963463), *EEFSEC* (rs2955117*)*, *AGTR2* (rs201226733), *WNT4 (*rs56318008*)*, *ADCY5* (rs4383453), and *RAP2C* (rs200879388) genes are associated with the duration of gestation, and variants in the *EBF1* (rs2963463), *EEFSEC* (rs201450565), and *AGTR2* (rs201386833) genes are associated with PTB risk (Zhang et al. 2017). Another GWAS study on 1490 African-American women found that insertion/deletion polymorphisms in the *PTPRD* gene interact with the degree of maternal stress in a dose-responsive manner and increase the risk of PTB (Hong et al. 2021). However, almost equal numbers of GWAS (conducted on the maternal or fetal genome) failed to find a significant association with genetic polymorphisms after multiple comparisons or Bonferroni correction or due to the failure to replicate in validation cohorts (Rappoport et al. 2018; Wu et al. 2013; Zhang et al. 2015). Irrespective of many shortcomings, these GWAS studies had an advantage over the traditional candidate gene approach because of their large sample size and the fact that they simultaneously search for hundreds of common genetic variants associated with the disease risk. This approach is also free of hypothesis bias and can lead to the identification of novel genetic variants and potential mechanisms involved in disease risk (Dolan and Christiaens 2013).

14.3.3 Epigenetic Factors

In addition to the genetic variants, the role of epigenetic changes in PTB risk cannot be ignored. The fetus is continuously exposed to varying intrauterine environments according to the mother's lifestyle and genetic makeup. Adverse maternal exposures alter the intrauterine environment and can affect epigenetic programming during the developmental period of the fetus (Gunasekara et al. 2019; Kaminen-Ahola et al. 2010). Recent studies have shown that maternal heavy metal (cadmium) exposure changes DNA methylation status in the cord blood and is associated with PTB risk

(Koh et al. 2021; Yang et al. 2016). A comprehensive meta-analysis has shown that prenatal maternal smoking is significantly associated with differential DNA methylation in the newborn's blood, and these changes persist in later childhood (Joubert et al. 2016). Several other epigenome-wide association studies (EWASs) confirm the association of maternal exposure to traffic-related air pollution, particulate matter, heavy metals (like lead, mercury, and arsenic), and maternal folate levels with changes in the methylation status of cord blood (Argos et al. 2015; Cardenas et al. 2017; Gruzieva et al. 2017, 2019; Joubert et al. 2016; Salnikow and Zhitkovich 2008; Wu et al. 2017). The role of maternal exposure in adverse birth outcomes is evident from these studies; however, the exact mechanism of epigenome-mediated disease risk still remains unclear (Park et al. 2020).

14.3.4 Vaginal Microbiome and PTB Risk

It is a well-established fact that inflammation plays a significant role in the pathophysiology of PTB. Microorganisms can ascend from the vaginal microbiome into the intrauterine cavity and release endotoxins, resulting in the activation of inflammation-mediated pathways and causing intrauterine infections (Lee et al. 2017). Lactobacillus species are predominant among the vaginal microbiota in the majority of healthy pregnant women, and a drastic decrease in Lactobacillus spp. number can disturb the vaginal microflora balance, resulting in an increased number of anaerobic microorganisms, also known as bacterial vaginosis (Subramaniam et al. 2016). Studies have shown that the occurrence of bacterial vaginosis is associated with an increased risk of preterm birth (Nelson et al. 2014; Ugwumadu 2002). African-American women are more likely to have a diverse vaginal microflora and also have a higher incidence of bacterial vaginosis compared to European women, hence a higher risk of PTB (Culhane et al. 2002; Fettweis et al. 2014; Kramer and Hogue 2008). However, contradicting results have also been published showing that in African-American and Hispanic women, high mycoplasma and low bacterial vaginosis-associated bacteria-3 (BVAB3) were associated with preterm birth risk (Wen et al. 2014). A few studies have also shown no significant difference in the vaginal microbiome among women delivering spontaneous preterm and term babies (Hyman et al. 2014; Romero et al. 2014). Despite contradicting results, the role of ethnicity or race cannot be undermined as suggested by the above studies; however, these disparities cannot be explained alone due to sociodemographic, cultural, or lifestyle differences, and more studies with a larger sample size need to be undertaken to delineate the exact mechanism. Gene–environment interactions also play an important role in susceptibility to PTB when describing microorganisms-related infections during pregnancy. Studies by Macones et al. (2004) and Gómez et al. (2010) have shown that mothers with a susceptible genotype in any of the *TNF-α, PRKCA, FLT1*, and *IL-6* genes increase the risk of PTB in the presence of bacterial vaginosis as compared to women who have the same genotype but have no bacterial vaginosis (Gómez et al. 2010; Macones et al. 2004). Another study showed that certain maternal and fetal gene variants involved in inflammation

regulation increase the risk of PTB when mothers have vaginal or urinary tract infections (Elias et al. 2021).

14.4 Genetic Testing in Pregnancy for PTB Risk

The use of genetic factors in the diagnosis of PTB risk has long been debated. As mentioned above, enough data are available to suggest that women with increased PTB risk have familial/personal obstetric history as well as racial disparity, indicating the role of genetic factors (Fig. 14.2). The majority of these studies imply the role of maternal genetic factors contributing to PTB risk. Almost 25% of genetic variance in PTB can be explained by maternal genetic factors, 5% by fetal factors, and 18% by environmental factors (Svensson et al. 2009). A few studies have also recommended the use of biological markers such as *IL-6* and *TNF-α* levels in maternal serum; *MMP-8, IL-6, IL-8,* and *TNF-α* in amniotic fluid, fetal fibronectin in cervicovaginal secretions as biomarkers of PTB risk (Foulon et al. 1995; Nien et al. 2006; Ville and Rozenberg 2018; Vogel et al. 2007). *TNF-α* (−308A) polymorphism was also suggested to be a genetic biomarker for PTB risk; however, the analysis failed to show the shift in the probability of PTB {*TNF-α*(−308A), likelihood ratio: 1.2 (95%CI: 1.1–1.5)} (Hee 2011).

Many studies have reported interesting genetic variants in mothers and infants in inflammatory, immune, developmental, and other biological pathways of importance; however, the available data are insufficient to suggest genetic testing for assessing the risk of PTB. Most of the genetic factors have been identified by association studies (candidate gene and GWAS), and considering the multifactorial

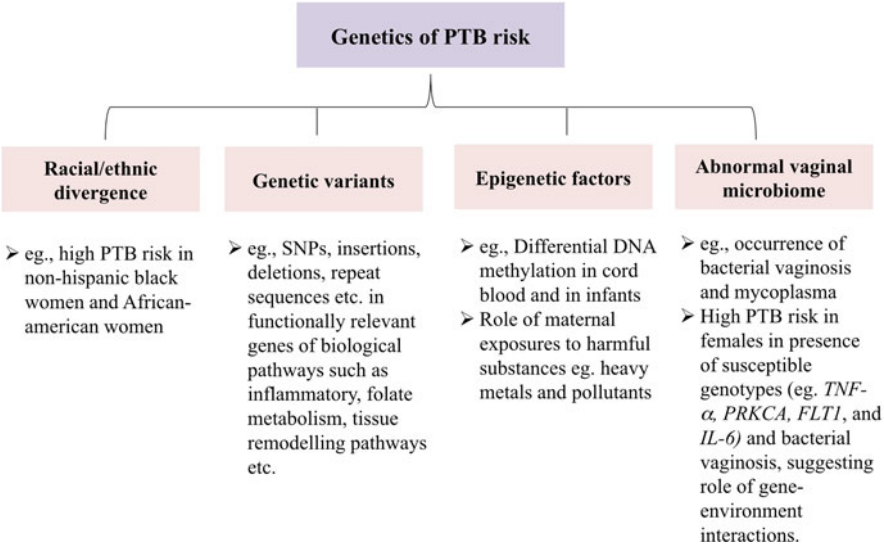

Genetics of PTB risk
Racial/ethnic divergence
Genetic variants
Epigenetic factors
Abnormal vaginal microbiome
eg., high PTB risk in non-hispanic black women and African-american women
eg., SNPs, insertions, deletions, repeat sequences etc. in functionally relevant genes of biological pathways such as inflammatory, folate metabolism, tissue remodelling pathways etc.
eg., Differential DNA methylation in cord blood and in infants
Role of maternal exposures to harmful substances eg. heavy metals and pollutants
eg., occurrence of bacterial vaginosis and mycoplasma
High PTB risk in females in presence of susceptible genotypes (eg. TNF-α, PRKCA, FLT1, and IL-6) and bacterial vaginosis, suggesting role of gene-environment interactions.

Fig. 14.2 Genetic factors and the risk of preterm birth

etiology and scanty data available on the genetics of PTB, it would be premature to highlight the significance of particular genes/alleles/variants for genetic testing. Most of these studies lack a sufficient sample size and have not ruled out confounding factors, such as ethnicity, diversity, nutrition, and lifestyle factors. Large prospective studies need to be conducted to identify useful genetic biomarkers of clinical relevance. Contrary to this, genetic factors alone do not explain its etiology, and gene–environment interactions need to be studied more extensively in the future.

14.5 Conclusions and Future Perspective

Gestational duration is determined by both maternal and fetal factors. Most of the studies suggest the role of genetic influence in the occurrence of PTB; however, the role of environmental factors and genetic relatedness, especially in twin or familial studies, cannot be underestimated. There is an increased risk of maternal predisposition due to certain genetic variations or family/obstetric history, but there are numerous fetal genetic and epigenetic signatures that can change throughout gestation as a result of maternal exposure. Epigenetic modifications during early life can affect gene expression and impact the overall development of the fetus, resulting in a stressful intrauterine environment. In response to stress stimuli, fetal signals can trigger biological pathways involved in parturition. The intrauterine environment can also be affected as a result of certain environmental stimuli, such as bacterial vaginosis and urinary tract infections. It is apparent from the studies mentioned above that certain genetic variations are more harmful in the presence of specific environmental conditions. Therefore, genetic factors play a certain and significant role in PTB occurrence; however, our understanding of these factors is currently insufficient to identify genetic biomarkers for assessing PTB risk.

A few studies have shown that increased paternal age, black race, paternal inflammatory diseases, lack of paternal support during pregnancy, drinking and smoking, and preconceptional paternal exposure to parabens are associated with an increased risk of preterm birth (Bengtson et al. 2010; Chen et al. 2011; Mustieles et al. 2020; Palomar et al. 2007; Surkan et al. 2019; Zhu et al. 2005). One study has also reported that the risk of spontaneous early PTB is increased (OR: 1.6, 95% CI: 1.1–2.4) in infants of consanguineous parents (Mumtaz et al. 2010). However, additional research into paternal genetic variables linked to PTB risk is still needed. In the future, thorough research employing a range of samples (maternal, paternal, and fetal) will be necessary to investigate the function and relationship of genetic variables with PTB risk. It will advance our understanding about the risks associated with adverse pregnancy outcomes and pave the way for genetic testing to predict PTB risk.

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Reproductive Carrier Screening

15

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Abstract

People carry varying burden of genetic diseases, like recessive disorders. Therefore, the disease alleles they carry and their potential to pass them on to the future generations must be carefully screened. Reproductive carrier screening aims to educate individuals about their carrier status and the likelihood of passing the disease-causing genes to their progeny so they can make informed reproductive decisions. Genetic testing started in the 1970s with screening for Tay–Sachs disease in Jewish populations. Later, screening was extended to many diseases like thalassemia, cystic fibrosis, muscular dystrophy, and fragile X syndrome. Apart from Tay–Sachs disease, people with consanguineous history are also being targeted as they are more prone to genetic diseases. In this chapter, we have discussed reproductive carrier screening for diseases like hemoglobinopathies, fragile-X syndrome, spinal muscular dystrophy, cystic fibrosis, and diseases commonly seen in consanguineous marriages. We have focused mainly on preconception studies for these diseases and the importance of screening at this stage. We have also discussed the challenges in its implementation, such as issues related to societal, ethical, technical, and public awareness.

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Carrier screening · Premarital screening · Consanguineous marriage · Hemoglobinopathies · Cystic fibrosis · Fragile X syndrome · Spinal muscular atrophy, Genetic testing, Reproductive medicine

15.1 Introduction

An anomalous form of a gene, if expressed, may lead to medical conditions commonly known as genetic disorders. There are different categories into which these genetic abnormalities fall, for instance, deletions, duplications, substitutions, and repeat-length polymorphisms (https://medlineplus.gov/geneticdisorders.html). Genetic abnormalities are rare but heritable, thus raising concern for the upcoming generation. Autosomal recessive disorders (AR) is a subset of genetic abnormalities that can be passed down through families. By 2020, more than 200 recessive disorders had been described with a substantial disease load of 1.7–5 in 1000 newborns, in contrast to 1.4 in 1000 for autosomal dominant disorders (Xiao and Lauschke 2021). According to the Clinical Genome Database (CGD) of the National Human Research Institute, approximately 1875 protein-coding genes are known to be linked to recessive disorders.

A higher prevalence of recessive diseases is often seen in endogamous populations or genetically isolated populations. For example, a higher frequency of sickle cell disease in the malaria-endemic region (10–20 per 1000 individuals) and an increase in recessive disorders in countries like Saudi Arabia (7 per 1000 individuals) are well known (Antonarakis 2019). Due to an increase in the recessive disease burden, it is important to know the major risk variants in a population to reduce morbidity and long-term health outcomes (Xiao and Lauschke 2021). It has been reported that out of 7028 Mendelian disorders, 1139 are recessive; however, they should not be underestimated just because the true prevalence of these diseases is yet to be determined (Rowe and Wright 2020).

Genetic testing, such as carrier screening, came into existence in the 1970s for recessive diseases like Tay–Sachs in Ashkenazi Jews and thalassemia in Mediterranean tribes. By the end of the 1990s, experts recommended genetic screening of all individuals or couples in their preconception stage for cystic fibrosis (Kraft et al. 2019; https://ranzcog.edu.au/RANZCOG_SITE/media/RANZCOG-MEDIA/Women%27s%20Health/Patient%20information/Carrier-Screening-1st-Edition.pdf?ext=.pdf). Carrier screening involves different processes from sample collection to consent to analysis and interpretation of the results for reproductive choices (Fig. 15.1). There are different stages at which screening can be performed, but as the level progresses, there is a reduction in reproductive choices and the probability of healthy offspring if tested positive (Rowe and Wright 2020). Thus, to take full advantage of diverse reproductive choices, it is preferred that screening be done before pregnancy or in the early stages of pregnancy (Fig. 15.2). A common practice is being adopted for early-stage screening, such as in high school (high school carrier

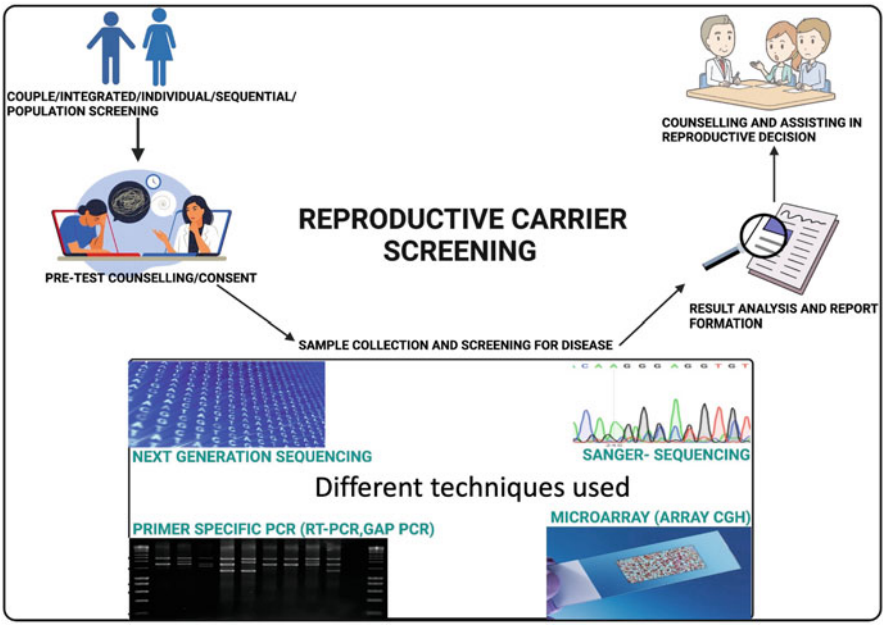


Fig. 15.1 The process of carrier screening

screening) (Ioannou et al. 2010) or premarital carrier screening (Dor Yeshorim program) (Singer and Sagi-Dain 2020), for diseases such as sickle cell anemia, cystic fibrosis, thalassemia, spinal muscular dystrophy, Tay–Sachs disease, and hemophilia (Table 15.1). Screening should become a common offering throughout the population in their early reproductive age rather than being specific to a population or region for a healthy future of the planet, removing stigmatization of testing positive and the need for a risk identifier group (Burke et al. 2011).

15.2 Premarital/Preconception Screening

In order to improve the short-term and long-term outcomes of the mother and child's health, there is a crucial need for optimizing preconception health. Preconception testing can be done with the help of general practitioners by creating awareness among women of reproductive age. Furthermore, doctors can assist couples in finding modifiable as well as nonmodifiable preconception risks and counselling them about family planning. Such testing helps in spreading knowledge about genetic diseases (sickle cell anemia, thalassemia), raising awareness about healthy marriages, preventing further social and psychological family problems, and therefore providing moral support to families with defective children. Several countries, such as the United Kingdom, the United States, Australia, Cyprus, Israel, Iran, Italy, Malaysia, the Netherlands, and Saudi Arabia, have adopted and welcomed

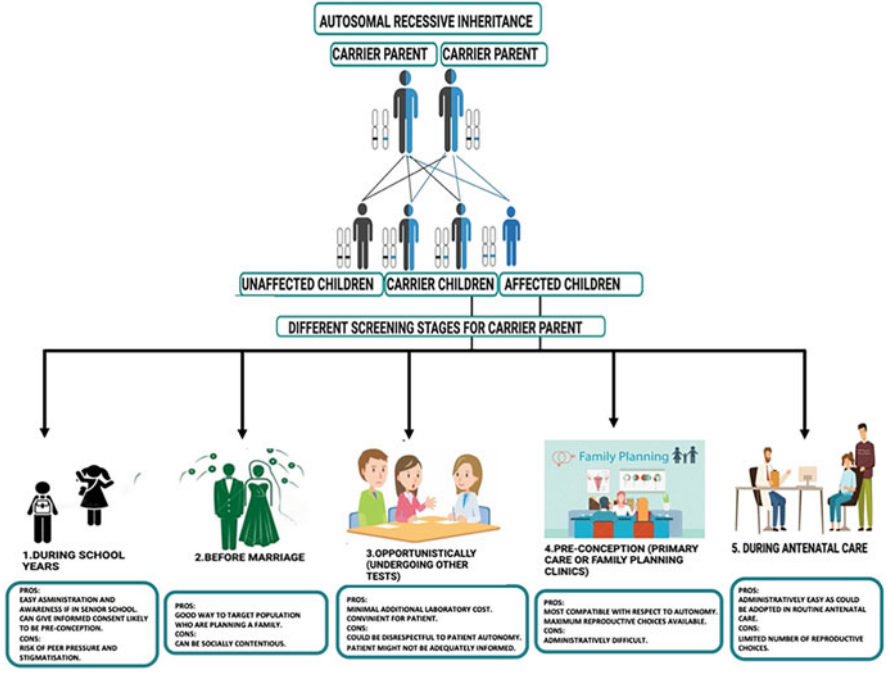


Fig. 15.2 Different stages of carrier screening

Table 15.1 Common carrier diseases and techniques used for genetic diagnosis

Disease	Carrier frequency	Variant screened	Techniques used
Thalassemia (Hemoglobinopathies)	Mediteranian-1 in 20 Hispanic- 1 in 13 Asian- 1 in 30	SEA/αα (α^0/α) $\alpha^{3.7}$/αα(α+/α) $\alpha^{4.2}$/αα(α+/α)	Gap PCR, MLPA (multiplex ligation dependent probe amplification) RT-PCR, Oligonucleotide array, Primer specific amplification, RT-gap-PCR, Array comparative genome hybridization (aCGH), NGS
Fragile X syndrome	1 in 250	45–54 CCG repeats (premutation) 55–200 CCG repeats (minor chance of disease) >200 CCG repeats (diseased)	PCR, Southern blotting, NGS (targeted miRNA)
Cystic fibrosis	Caucasian- 1 in 28 Ashk Jewish- 1 in 28 Hispanic- 1 in 46 Asian- 1 in 87	p. Arg334Trp p. Arg 347Pro p. Arg 347 his p. Phe 508 del p. Val 520 Phe p. Gly 542 X	Droplet digital PCR, sanger sequencing, NGS
Spinal muscular dystrophy	1 in 50	SMN1/5q 11.2–13.3 (SMA1,2,3) 12q 23–24 (congenital SMA) Deletion and intragenic mutation of exon 7 SMN2 copy number	RFLP, DNA sequence analysis (NGS), MLPA, QF-PCR (qualitative fluorescent PCR)
Tay–Sach (consanguineous)	Ashk Jewish- 1 in 27 General population- 1 in 300	c.1214-1215del AAinsG* c.1204-1206del c.759-774dup* c 1292 G > A	PCR, microarray, NGS

reproductive carrier screening (Delatycki et al. 2020). Due to the varying geographical prevalence of various diseases, countries only target those diseases with a high frequency in their populations. For example, Tay–Sach Disease (TSD), which results in the degeneration of the central nervous system, has a high prevalence in the

Australian Jewish community, for which preconception screening is being undertaken (Lew et al. 2011). In this study, it was found that 1 in every 25 Ashkenazi Jews is a carrier of TSD. Along with TSD, diseases like cystic fibrosis, Canavan disease, Bloom's syndrome, and Niemann–Pick disease type A were also screened in Jewish high school girls in Australia (Ioannou et al. 2010). It was projected that reproductive carrier testing of 1300 genes would be done in 10,000 Australian couples by the end of 2022.

Countries like Cyprus, whose population is the highest carrier of beta-thalassemia, started population screening in 1973 through hematological indices and hemoglobin electrophoresis, focusing on high school students and relatives of affected individuals. The country focused on building more effective screening strategies for individuals of reproductive age and providing a premarital certificate to them by 1980. Prepregnancy carrier screening for the five most common conditions has been undertaken in genetically isolated people in the northwest Netherlands since 2012. This group was targeted due to consanguineous marriages within the population, which resulted in children being affected by one of the five childhood disorders common in this population (Delatycki et al. 2020). The preconception screening aids in the detection of common genetic conditions like thalassemia, spinal muscular dystrophy, cystic fibrosis, hemophilia, and sickle cell anemia, which are discussed in depth in this chapter.

15.3 Hemoglobinopathies

Hemoglobinopathies are the most common among single-gene disorders. Population screening for diseases like α, β-thalassemia, and sickle cell disease (SCD) was initiated in neonates, reproductive-age adults (preconceptionally), and carrier families by governments and other organizations. Switching between Hb gene subtypes by on–off expression of α globin gene cluster and β gene cluster occurs throughout the developmental stages. Point mutations, insertions, and deletions in the α or β genes result in α-thalassemia and β-thalassemia, respectively, while structural variations in Hb due to amino-acid substitution in the β gene cluster give rise to sickle RBC.

According to a global epidemiology study, 5.2% of the world's total population is a carrier for hemoglobinopathy genetic variants (Modell and Darlison 2008). Out of 330,000 defective births, SCD contributed 83% while thalassemia contributed 17% (Goonasekera et al. 2018). Screening in high-prevalence areas is recommended at different stages, including adolescence (high school screening), reproductive-age females and males, and carrier female partners (premarital, preconceptional, or antenatal). Thalassemia is prominent in the Mediterranean region, the Middle East, and India. Furthermore, it is common in populations of African descent. Migration has also spread this disease to places like South and North America and Northern Europe (Cao and Kan 2013), suggesting screening even in those populations.

In 1970, screening for sickle cell anemia took place for the first time in Virginia, Canada, Greece, and Italy, which was later adopted nationwide (Alswaidi and

O'brien 2009). In a developing country like India, premarital screening for sickle cell disease was conducted, revealing that 30% of the participants and their family members were aware of preconception screening for sickle disease (Cousens et al. 2010). Different countries started screening for hemoglobinopathies, including the Mediterranean basin, followed by Greece and Cyprus, which initiated thalassemia screening due to consanguineous marriages. The Maldives made it compulsory for its entire population to screen for thalassemia. In 1975, Italy (Sardinia) began a thalassemia prevention program by screening premarital and antenatal groups. This test in late 1995 reduced the incidence of thalassemia from 1:250 to 1:4000 (Cousens et al. 2010). In Malaysia, Singapore, and Thailand, hematological tests are being undertaken to screen for thalassemia in premarital screening programs (Delatycki et al. 2020). A study based on Saudi Arabia's premarital screening 6-year data on hemoglobinopathies concluded that the frequency had reduced to a great extent, which in the future will further decrease the burden of these diseases (Memish and Saeedi 2011; Gosadi et al. 2021; Al-Qattan et al. 2019). Israel offers beta thalassemia free screening through its public health system to various ethnic groups, which helps in a 2/3 reduction of thalassemia-born babies per year (Metcalfe 2012). While initially endemic, these diseases have spread across the globe due to the migration of people; therefore, guidelines have been issued for carrier screening by the WHO (Old and Harteveld 2017).

Different techniques were adopted in the early screening strategy to identify the Hb pattern with the help of acid agarose or citrate agar gel, cellulose acetate electrophoresis, capillary zone electrophoresis, isoelectric focusing, automated capillary isoelectric focusing, and HPLC. Several advanced techniques employed later included Gap-PCR, MLPA (Multiplex Ligation-dependent Probe Amplification), RT-PCR, oligonucleotide microarray, primer-specific amplification RT-gap-PCR, and array comparative genomic hybridization (aCGH) (Old and Harteveld 2017). Screening for sickle cell disease can be done by sickling RBC due to insoluble polymer in the peripheral blood smear, sickle solubility test, etc. (Hamali and Saboor 2019). The advent of next-generation sequencing and gene chips with specific mutations helped screen a large number of mutations simultaneously (Goonasekera et al. 2018; Achour et al. 2021).

The success of screening in all countries and areas depends on its acceptance by the population and the awareness created by health officials, including counseling (Goonasekera et al. 2018). There are different challenges as the time of screening is delayed. A survey of 40 couples who had premarital thalassemia screening and counseling revealed that it was unethical to end the marriage if a screening revealed a positive result. Thus, there are lots of social and ethical values, general awareness, and counseling that need to be taken care of to bring such practices into existence (Algiraigri 2021; Traeger-Synodinos and Harteveld 2017).

15.4 Fragile X Syndrome (FXS)

FXS is one of the X-linked genetic conditions causing intellectual and developmental impairments due to an increase in the number of triplet CCG repeats in the Fragile X mental retardation (FMR1) gene on chromosome X. The normal range of CCG repeat is 6–44, which if increased to 55–200 is categorized as premutation, while its expansion to >200 leads to the full expression of the disorder. The whole mutant allele impairs brain development and synaptic function by lowering the FMRP protein, which is connected to the pathways regulating protein translation at synapses (Hill et al. 2010). The incidence and risk of high severity are quite higher in males as compared to females. In the USA, FXS hits 1 in every 4000 males and 1 in 6000–8000 females, but carrier females are four times higher than males (1 in 250 females and 1 in 1000 males) (Turner et al. 1996; Murray et al. 1996; de Vries et al. 1998; Crawford et al. 2001; Sherman et al. 2007). Due to the higher number of carrier females, it is very important to test women at their reproductive age (Hill et al. 2010; https://rarediseases.org/rare-diseases/fragile-x-syndrome/). Many countries have adopted and initiated screening programs for FXS. For example, Spain, on screening 3413 pregnant and 318 nonpregnant females on Balearic Island, found that one in 106 females was a premutation carrier (Alfaro et al. 2016). In Australia, the cystic fibrosis screening program in 2012 also included screening for fragile X syndrome and found that every 1 in 20 tested positive for being a carrier for the FXS condition (Delatycki et al. 2020). However, an expanded carrier screening that identified 122 FMR1 premutation carriers found that 98% opted for pretest counseling. Among these counseling patients, 74% supported the expansion of carrier screening for women planning for pregnancy in their reproductive age to reduce the risk of pregnancy (Johansen Taber et al. 2019).

To develop a model for the future screening program, Metcalfe et al. (2008) screened nonpregnant women for being carriers for FXS. Their study was based on pre–post screening questionnaires and evaluated women ($N = 65$) who participated in the test had a positive attitude and supported the preconception screening idea (Metcalfe et al. 2008). Studies were also conducted to evaluate the awareness and reaction of women to being carriers for FXS. Women stated diverse responses, such as the families of the affected children found it difficult to give an opinion regarding screening due to their attachment to their children, while others stated that the timing of screening is important (Anido et al. 2005; Archibald et al. 2009). Further, 9459 Israeli women in the preconception and prenatal stages who had no family history underwent screening. It identified that out of 80% of the antenatal participants, 134 were carriers of premutations. Seeing a high frequency of premutations, it was concluded that preconceptional screening must be done (Pesso et al. 2000). Routine testing includes regular cytogenetic testing, where DNA analysis either by Southern blot or PCR to identify the mutation with a different number of repeats along with the status of the methylation in full mutation is recommended in suspected individuals (Taylor 1994; Saldarriaga et al. 2014). In conclusion, looking at the incidence of the disease, it is advisable to consider preconception screening in high-prevalence areas to decrease the disease burden.

15.5 Cystic Fibrosis

Cystic fibrosis (CF) stands out on the list of diseases that need to be majorly monitored premaritally or preconceptionally due to the heavy burden of care required by affected children and the compromised quality of life it brings to the family. CF is a result of genetic variation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. To date, more than 2111 variations have been identified in the CFTR gene (http://www.genet.sickkids.on.ca/StatisticsPage.html). The prominent phenotypic characteristics are chronic sinopulmonary disease and salt loss syndrome. Numerous guidelines and recommendations emphasize carrier screening of populations preconceptionally. However, screening adaptation by the general public is not that easy, as most of them consider it to be of no use to them. A maximum number of participants (87%) in the CF screening program in the United Kingdom were from family planning clinics, as they took screening to be relevant, and the rest of the participants (66%) were from general clinics, and they were least interested in participating in screening (Metcalfe 2012).

CF was added to the screening program of Israel's Ashkenazi Jews in 2008 and was sponsored by the Ministry of Health due to its higher frequency (1 in 60) in the population, as reported by the Association of Israeli Medical Geneticists (Zlotogora 2019). According to Kornreich et al. (2004), Ashkenazi Jews usually prefer premarital screening for cystic fibrosis, where all new mutations are checked and older ones are monitored through PCR and restriction digestion, which helps them control the disease in the upcoming generations (Kornreich et al. 2004). Further analysis done by the Italian Cystic Fibrosis Research Foundation during an online public survey concluded that both the general public and medical workers think that CF should be included in reproductive carrier screening.

All couples who are of reproductive age should be offered CF screening using a mutation panel of 25 CFTR variations, according to a protocol established in 2001 by the American College of Medical Genetics and Genomics (ACMG) and the American College of Obstetricians and Gynecologists (ACOG) (Delatycki et al. 2020). In contrast to other countries, the Netherlands offered periconceptional ancestorial-based carrier screening only for couples that were planning for pregnancy and had an ancestral background of the disease. Lakeman et al. (2009), while screening for factors determining the uptake of cystic fibrosis screening, concluded that the choice of a reproductive decision should be promoted to have more couples take up the preconceptional marital screening (Lakeman et al. 2009).

A study that compared newborn screening with CF preconception carrier screening found that both have advantages and disadvantages, but that preconception carrier screening is a significantly safer procedure because it allows for more reproductive options before the conception of an infected child (Castellani and Massie 2014). Ioannou et al. (2014) presented a systematic review of 32 years of data on cystic fibrosis screening and noted the opinions of individuals at different stages of screening. Out of the screened individuals in the high school and college stages, 40–59% considered that preconception is the best time for screening. Among all nonpregnant women and couples who were screened, 69–89% believed that these

screenings should be routinely offered to those who are planning for pregnancy. Among pregnant women screened, 98% considered the preconception stage to be the best time for screening (Ioannou et al. 2014).

15.6 Spinal Muscular Atrophy

A disorder involving the central nervous system, peripheral nervous system, and voluntary muscle movement is called spinal muscular atrophy (SMA). Due to higher incidence and mortality rates in newborns, taking preventive measures has become necessary. The disease is categorized into three groups depending on the onset and course of the disease, of which type 1 is associated with severe disability and mortality in children up to 2 years of age (https://www.acog.org/womens-health/faqs/carrier-screening-for-spinal-muscular-atrophy).

It is a genetic condition that damages the neurons of the body that control muscles for breathing, swallowing, and limb movement due to genetic variations in the survival motor neuron (SMN1/2) gene. This gene is located on chromosome 5q13. There are nine exons in the SMN1 gene, and exon 7 deletion has often been reported as the cause of the disease (Prior et al. 2008). It is estimated that 1 in 50 people, regardless of history, is a carrier for the abnormal SMN1 gene. According to a genetics home reference, 1 in every 8000–10,000 individuals in the world is affected by SMA (https://www.medicalnewstoday.com/articles/192245#diagnosis). There are reports of SMA from all ethnicities. Therefore, it is advisable to go for premarital screening to avoid the occurrence of the disease (Prior et al. 2008; https://www.samitivejhospitals.com/article/detail/preconception-carrier-screening-for-spinal-muscular-atrophy-sma).

Another survey was designed in the United Kingdom to know the views about genetic screening from people having SMA. Overall, 77.2% of the surveyors (families with SMA patients and SMA patients themselves) were in support of preconceptional screening. Lawton et al. (2015), in a study on the Australian population, identified that 77% of the SMA participants of genetic screening supported preconceptional screening (Lawton et al. 2015). The diverse views on screening were related to their perception of life after SMA. The one with a low-quality life preferred the adoption of population-wide screening over the one with a high-quality SMA life (Boardman et al. 2017). As a large number of affected patients have opted for preconceptional screening, it is important to be included in reproductive screening programs.

15.7 Consanguineous Diseases

Traditional consanguinity is a problem that has been practiced for a long time and has become a major cause of recessive genetic disorders. One billion people globally prefer consanguineous unions, and those from the Middle East, West Asia, and North Africa are more likely to carry congenital diseases to their offspring due to this

trend. Thus, there is a need for public awareness regarding the devastating effects of consanguineous marriages and the importance of preconception screening in the primary healthcare system (Hamamy 2012). Sensorineural hearing loss is one of the most prevalent diseases in consanguineous cases, and the risk of having more than one kid with this condition within a consanguineous marriage is 3.5 times higher than the typical risk (Almazroua et al. 2020). Sensorineural hearing loss, also known as nerve deafness, occurs due to a mutation in the transmembrane channel-like 1 (TMC-1) gene (Ramzan et al. 2020). In a study on 950 children with hearing loss, 71% were from consanguineous marriage couples, and 27% of these cases had inherited hearing loss (El-Din and Hamed 2008). In cases like sensorineural hearing loss, deaf individuals must be supported in terms of premarital screening and counseling. To create awareness, there is a need for extra efforts to train the counselors as well as family members of the affected individuals.

A study on 64 deaf and hard-hearing female students intended to focus on their awareness and attitude toward premarital screening revealed that 64% of the participants were born out of consanguineous marriages. Regarding the knowledge of premarital screening and counseling, 81.3% of the participants had a positive attitude, which turned to 95.3% after educating them. However, one limitation of the awareness campaigns highlighted in the study was a lack of knowledge of sign language among health professionals, which makes this group the least targeted in educational and awareness programs (Zaien et al. 2021). Many other diseases are linked to inheritance because of consanguineous marriages, such as thalassemia and sickle cell anemia, which have already been discussed in previous sections of this chapter, and these are being screened before conception in consanguineous populations in Jordan, Saudi Arabia, Iran, Iraq, Bahrain, and Turkey. Screening becomes challenging in countries with traditional consanguinity, as it is hard to make them believe the demerits of their societal norms (Hamamy 2012).

Microcephaly, an abnormal brain development disorder, is also a result of consanguineous marriages and is quite prevalent in Iran, where out of 1202 microcephaly cases, 104 individuals were born out of consanguineous marriages. Due to the large number of inherited cases of microcephaly from consanguineous marriages, it is important to emphasize introducing early detection and clinical guidelines to decrease mortality (Saki-Malehi et al. 2017). Another study reported three deleterious mutations in consanguineous couples, which were premaritally screened for autosomal recessive disorders like microcephaly (CEP135), xeroderma pigmentosum (ERCC3), and reticular dysgenesis (AK2) (https://diagnostics.medgenome.com/stories/1372/). Xeroderma is a genetic disorder of decreased DNA repair with symptoms of change in skin pigmentation and nervous system problems, whereas reticular dysgenesis is a combined immunodeficiency syndrome that shows agranulocytosis, lymphopenia, and lymphoid-thymic hypoplasia (https://diagnostics.medgenome.com/stories/1372/). Due to the numerous diseases that might arise from consanguineous marriages, adopting reproductive carrier screening in consanguineous communities is imperative.

15.8 Advancement in Reproductive Carrier Screening

Carrier screening was earlier undertaken only for people with a history of the disease or in tribes with a high frequency of the disease. One such disease is Tay–Sach syndrome, which aggregates in Ashkenazi Jews. The absence of this enzyme causes fat deposition in the nerve cells (https://www.ncbi.nlm.nih.gov/books/NBK564432/). However, this ethnic-based screening cannot be used to identify all people at risk, as only a single panel of sequence variants is considered for testing. All these hurdles were surpassed with the advent of next-generation sequencing, which helps in detecting a larger set of sequence variants and cover a much larger number of diseases without much increase in cost or time, due to which the term carrier screening was replaced by expanded carrier screening (ECS), although many challenges need to be overcome by expanded carrier screening too (Kraft et al. 2019).

The Royal Australian College of General Practice advises that regardless of family history or ethnicity, all women planning a pregnancy or in the early stages of pregnancy within a population should undergo expanded carrier screening for a minimum of the four most prevalent genetic diseases: thalassemia, CF, SMA, and FXS. In the Netherlands, ECS is being provided by the government since 2016 to interested couples; however, it covers only 50 diseases (Schuurmans et al. 2019). In Israel, carrier screening for thalassemia (Arabs, Druze, and Jews), CF, and TSD (Ashkenazi Jews) is being undertaken separately, whereas depending on the ethnicity of the couples, screening for CF, SMA, and FXS is also being conducted in the whole population through expanded carrier screening depending on the ethnicity of the couple (Zlotogora et al. 2016). Expanded carrier screening was also done for the diseases that had a frequency of 1 in 1000 or more in Arabs and the Druze community of Israel (Zlotogora et al. 2016). Expanded carrier screening in China for 11 monogenic diseases was done on 10,476 couples, out of which 27.5% were carriers for at least 11 conditions without any family history (Zhao et al. 2019). However, many countries have yet to adopt expanded carrier screening as a routine practice, including switching from traditional carrier screening to expanded carrier screening. For example, in Saudi Arabia, several diseases like CF, hemoglobinopathies, and consanguineous diseases are being premaritally screened, but steps are being taken for an expanded premarital screening program (Delatycki et al. 2020).

15.9 Challenges in Reproductive Carrier Screening

The challenges in reproductive carrier screening have different levels and vary across patients, communities, and countries, depending on various factors. Some countries have worked hard to create awareness and make genetic screening more acceptable. Challenges have different forms, from taking the test to taking up counseling, including the appropriate time for such testing. Since it is still difficult to conduct screening of the entire population for a particular disease, the most

challenging aspect is identifying the target population due to the lack of symptoms of the disease.

At the molecular level, the challenge is to cover intronic regions in the screening panel, as introns form the major part of the human genome, and neglecting them during genetic screening is a risky process. Diseases might have resulted from deletions in the splicing site or splicing defects along with mutations in the intronic regions. Familial hemophagocytic lymphohistiocytosis (FHL) is an autosomal recessive disorder for which a mutation in the intronic region 1 within the UNC13D gene was found to be responsible (Meeths et al. 2011), suggesting that the intronic regions cannot be excluded from screening. The other molecular challenge faced during cystic fibrosis counseling is the interpretation of CFTR variants and their complicated molecular mechanisms, which result in complex and unpredictable phenotypes.

As opposed to other populations of Asians and Americans, non-Hispanic Caucasians do not have a significant number of CF pathogenic mutations. However, screening programs target only known variants that have been studied in the past (Kraft et al. 2019). While other challenges include literacy rates, consanguineous marriages, social norms, the genetic understanding of counselors or healthcare professionals, and awareness. These challenges have been discussed in Chap. 20 of this book.

15.10 Conclusions and Future Prospective

Recessive disorders have significantly increased the probability of disease burden in the upcoming generations, making it crucial to understand the key risk factors in a community in order to lower morbidity and improve long-term health outcomes in the newborn. Screening at reproductive age helps in making better reproductive decisions with the aim of decreasing the future challenge of the anticipated disease load. Reproductive carrier testing for prominent genetic diseases, such as thalassemia, spinal muscular dystrophy, cystic fibrosis, hemophilia, and sickle cell anemia, is already in practice. Different countries have adopted reproductive carrier screening for diseases like cystic fibrosis, thalassemia, and sickle cell disease in their populations, which has resulted in healthy reproductive decision-making. Among the screened groups, people with consanguineous marriages have contributed significantly to recessive genetic illnesses. Thus, adopting reproductive carrier screening in consanguineous communities is essential due to the multiple diseases that could result from consanguineous marriages.

There are different techniques, such as Gap-PCR, MLPA (Multiplex Ligation-dependent Probe Amplification), RT-PCR, oligonucleotide microarray, primer-specific amplification RT-gap-PCR, and array comparative genomic hybridization that have helped in understanding the genetic contribution to reproductive carrier testing. The advent of next-generation sequencing has accelerated the process of carrier screening. Next-generation sequencing has helped in detecting a larger set of sequence variants and covering a much larger number of diseases without much

increase in cost or time, due to which expanded carrier screening came into existence. However, many countries have yet to adopt expanded carrier screening as a routine practice, including switching from traditional carrier screening to expanded carrier screening. Expanded carrier screening must be promoted in order to cover a much larger number of variants, which will help in finding new variants associated with the diseases and covering many diseases in one go.

At the same time, there are many hurdles (ethical, social, general awareness, and counselling) associated with reproductive carrier screening that should be taken into serious consideration to reduce the number of prevalent diseases. We conclude that for a healthy future, carrier screening should be made available to everyone in their early reproductive years, eliminate the stigma associated with testing positive and the requirement for a risk identification group.

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Genetic Testing in Endometrial Cancer

16

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Abstract

Endometrial carcinoma is one of the most commonly occurring gynecological malignancies. It is diagnosed mostly at the age of 50, yet 5% of cases belong to females of reproductive age. Owing to its wide classification on various parameters such as histology, microscopic features, pathogenesis, and prognosis, there is no well-established method to screen endometrial carcinoma at present. However, NGS and microarray-based tools alone or in combination with traditional techniques corresponding to morphological tumor identification could provide early and precise diagnosis. An amalgamation of genetic counseling with clinical and prognostic assessment could possibly serve to improve the diagnosis of endometrial carcinoma. This chapter sheds light on the etiology of endometrial carcinoma and genetic testing based on altered patterns of genes responsible for its establishment and progression. It gives valuable insights into commercially available panels for reproducible and accurate prediction of cancer risk.

Keywords

Endometrial carcinoma · Endometrial cancer · Genetic testing · Genetic diagnosis · Reproductive medicine · Dysfunctional uterine bleeding · Infertility

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16.1 Introduction

Endometrial cancer (EC) is the sixth most commonly diagnosed cancer in women, with 150,000 new cases identified annually worldwide (Coll-de la Rubia et al. 2020). Around 90% of the EC cases are sporadic, and the remaining 10% are familial (Okuda et al. 2010). WHO has classified endometrial cancer into nine distinct classes: endometrioid carcinoma, serous carcinoma, serous endometrial intraepithelial carcinoma, mucinous carcinoma, clear cell carcinoma, neuroendocrine tumor, undifferentiated carcinoma, mixed cell adenocarcinoma, and dedifferentiated carcinoma. About 85% of total endometrial cancer cases show endometrial carcinoma, and about 3–10% of cases show serous carcinoma (Low et al. 2010). In 1983, Bokhman categorized EC into two broad groups of tumors; estrogen-dependent endometrioid endometrial carcinomas (EECs) or type I, and non-endometrioid endometrial carcinomas (NEECs) or type II tumors (Bokhman 1983). The etiology of type I tumors or EECs is associated with estrogen-related pathways and arises from unopposed estrogen stimulation. In contrast, type II tumors, or NEECs, are estrogen-independent, consisting mostly of serous carcinomas (Okuda et al. 2010). Type I constitutes around 80% of the endometrial cancers, serous carcinomas comprise around 10%, and the rest 10% comprise clear-cell, squamous cell, and mixed adenocarcinomas (Amant et al. 2005). Type I includes low-grade (1 and 2) tumors that tend to appear in premenopausal and younger postmenopausal women, whereas type II includes high-grade (grade 3) tumors, which are frequently observed in older postmenopausal women. Type II has a relatively poor prognosis.

The risk factors associated with the development of endometrial cancer include long-term exposure to unopposed estrogens, early menarche (Xu et al. 2004), late menopause (Karageorgi et al. 2010; Yang et al. 2013), obesity, diabetes (Webb 2015), multiparity, and long-term use of tamoxifen as a treatment for breast cancer (Hu et al. 2015). An inverse correlation between cigarette smoking and endometrial cancer has been observed, probably due to a reduction in estrogen levels (Blakely et al. 2013). Furthermore, it is estimated that the first-degree relatives of individuals with endometrial cancer have an increased risk of the disease, with a relative risk (RR) of 1.8 (Lucenteforte et al. 2009). The familial aggregation of the disease suggests that genetic factors are key instruments in modulating the risk of endometrial cancer, ranging from the heritability of multiple low-risk cancer predisposition variants to single very high-risk variants.

16.2 Molecular Signaling Pathways and Associated Mutations in Endometrial Carcinoma

Endometrioid histotype is largely described by alterations in the canonical WNT–-β-catenin pathway, RAS-MEK-ERK pathway, PI3K-PTEN-AKT-mTOR pathway, high frequency of microsatellite instability (MSI) due to defects in the mismatch repair (MMR) system, and a reasonably high occurrence of POLE mutations linked

with an ultramutated phenotype (Bell and Ellenson 2019). Alterations in genes such as *FGFR2, CTNNBB1, PIK3CA, PIK3RI,* and *KRAS* have also been reported in endometrioid carcinoma (Byron et al. 2012).

16.2.1 WNT–β-Catenin Pathways

The canonical WNT–β-catenin pathway is particularly known to direct numerous biological processes, including cell proliferation, cell migration, cell survival, cell fate determination, and cell polarity. The gain-of-function mutations in the *CTNNB1* gene, resulting in the inhibition of phosphorylation and subsequent ubiquitin-mediated proteasomal degradation of β-catenin, are common features associated with the EECs. *CTNNB1* mutations occur in up to 37% of endometrioid carcinomas (Bell and Ellenson 2019). Many studies, including meta-analyses, have shown that mutations in exon 3 of CTNNB1 are associated with low-grade, early-stage EEC with significant recurrence risk (Liu et al. 2014; Travaglino et al. 2022). The common mutations are found to be missense in exon 3 within a stretch of 14 amino acids (codon 32–45) (Wright et al. 1999). These are positively correlated with β-catenin accumulation in the EECs. These mutations are associated with overexpression of members of Wnt, such as WNT5A, Sox9, FZD10, LEF1, and TGFB2 (Moreno-Bueno et al. 2002). CTNNB1 gene alterations are more prevalent in ovarian endometrioid carcinoma than in endometrial carcinoma; hence, they can be primarily utilized in clinical testing of ovarian carcinoma (D'Alessandris et al. 2021).

RNF43 (Ring Finger Protein 43) is a ubiquitin ligase that negatively regulates the WNT–β-catenin pathway by targeting the Wnt receptor Frizzled (FZD) (Serra and Chetty 2018, p. 43). RNF43 is somatically mutated in around 18–27% of endometrioid carcinomas (Bell and Ellenson 2019). SOX17 (SRY-Box Transcription Factor 17) transcription factor inhibits the activity of the Wnt/β-catenin pathway by negatively regulating β-catenin and TCF–LEF-induced gene transcription. Somatic mutations in the SOX17 gene are reported in 11.5% of endometrioid carcinomas (Bell and Ellenson 2019; Walker et al. 2017). The common missense mutations in the SOX17 gene are p.Ala96Gly and p.Ser403Ile (Walker et al. 2017). The CTNNB1, K-ras, and SOX17 genes have been placed in the Copy Number Low subgroup by the Cancer Genome Atlas (TCGA).

16.2.2 RAS-RAF-MEK-ERK Pathway

The RAS-RAF-MEK-ERK pathway is one of the major pathways that regulate cell proliferation, cell survival, and differentiation (Castellano and Downward 2011). Somatic mutations in the *KRAS* gene are one of the chief mechanisms for the activation of the RAS–RAF–MEK–ERK pathway in endometrioid carcinoma. *KRAS* mutations are described in 15–24% of endometrioid tumors overall, with mutations observed in around 18% of the EECs compared with 3% of the ECs (Bell

and Ellenson 2019). Most of the KRAS-mutated ECs also show alterations in the PTEN, PIK3CA, and/or PIK3R1 genes (Cheung et al. 2011; Konopka et al. 2011; Rudd et al. 2011; Urick et al. 2011). In a nutshell, these genes can be regarded as potential pathogenic biomarkers in endometrioid endometrial cancer.

16.2.3 PI3K-PTEN-AKT Pathway

The most commonly altered pathway in the EECs is the PI3K-PTEN-AKT pathway, which regulates cell proliferation, growth, and survival (Engelman 2009). Around 80% of EEC tumors are observed to have somatic alterations in the PI3K pathway. These alterations include variations in the *PIK3R1* (p85α), *PIK3R2* (p85β), *PIK3CA* (p110α), *AKT1,* and *PTEN* genes (O'Hara and Bell 2012). Several missense mutations have been found in PIK3CA exon 20 and a few in exon 1 (545D, H1065L, T1052K, T1025A, H1047L) (Konopka et al. 2011). The collective frequency of the PI3K pathway mutations is estimated to be around 39% [PTEN (13%), PIK3CA (35%), and PIK3R1 (8%)]. The mutations in the PI3K pathways are notably less frequent in serous ECs in comparison to EECs (O'Hara and Bell 2012).

16.3 Gene Mutations in Endometrial Carcinoma

16.3.1 *ARID1A* Mutations

ARID1A or AT-Rich Interaction Domain 1A is a tumor suppressor gene that codes for the BAF250a protein, which forms a core subunit of the SWI/SNF complex. ARID1A-containing SWI/SNF chromatin remodeling-complex regulates tumor suppression. Underexpression of nuclear ARID1A expression correlates to increased phosphorylation of the AKT gene (Wiegand et al. 2015). Dysregulated expression of ARID1A is reported in 26%–29% of low-grade (G1/G2) EECs and in 39% of high-grade (G3) EECs (O'Hara and Bell 2012). The frequency of mutations in this particular gene is high, which is often linked to increased antiapoptotic activity and cell proliferation. Mutations in ARID1A lead to gain of function of the *PIK3CA* oncogene and inactivation of the tumor suppressor PTEN in ovarian endometrioid carcinoma, elevating the cancer risk (Chandler et al. 2015). Low expression of BAF250a is detected in around 18% of serous ECs and 26% of clear cell ECs (Wiegand et al. 2011). There are very limited studies on *ARID1A* mutation screening in serous and clear ECs, and none of them has detected any mutation (Guan et al. 2011).

16.3.2 *TP53* (P53)

The TP53 gene encodes for tumor protein P53. This protein plays a crucial role in inhibiting the proliferation of cells with DNA damage. It happens to be the most

frequently altered cancer gene in serous ECs (46%) (O'Hara and Bell 2012). But frameshift or nonsense mutations are also present in a subset of EECs (15%). The exact mechanism resulting in the TP53 gene mutation is not well understood. In response to the DNA damage, the p53 protein tends to accumulate, resulting in cell cycle arrest and apoptosis. Functional mutations in the TP53 gene may lead to the accumulation of non-functional p53 protein, which may further aggravate the propagation of aberrant cells (Okuda et al. 2010). A positive immunostaining of p53 protein was observed in 80%–86% of serous tumors. Moreover, somatic mutations in the TP53 gene were observed in 53%–90% of tumors (Ambros et al. 1996; Lax et al. 2000).

16.3.3 PPP2R1A

The PPP2R1A gene encodes a constant regulatory subunit of protein phosphatase 2. Protein phosphatase 2 is one of the four major Ser/Thr phosphatases that negatively control cell growth and division. The trimeric holoenzyme consists of a scaffolding subunit A (PR65), a variable regulatory subunit B, and a catalytic subunit C (PP2Ac) (Eichhorn et al. 2009). The PPP2R1A and PPP2R1B genes encode for scaffolding subunits PR65α and PR65β, respectively. Mutations found so far are heterozygous missense located on the surface of the A subunit in the interaction interface between the A and B subunits. Somatic mutations in the PPP2R1A (PR65α) gene are frequently observed in serous carcinomas (17–41%) in comparison to EECs (5%–7%).

16.3.4 HER-2/ERBB2

ERBB2 is an oncogene that encodes a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases, which are involved in cell signaling. High gene dosage of HER-2/ERBB2 is frequently reported in serous ECs compared to EECs. An enhanced expression of HER-2/ERBB2 is detected in 17%–80% of serous ECs (O'Hara and Bell 2012; Okuda et al. 2010). Further studies are required to elucidate the exact mechanism and role of HER-2/ERBB2 in the pathogenesis of endometrial cancer. The mutations specified are mostly in L755S, 1767 M, D769Y, G815R, V842I, and T862A. Mutations found to be linked with endometrial serous carcinoma are V842I and H878Y (McMeekin et al. 2016).

16.4 Genetic Testing in Endometrial Cancer

It is now well established that hereditary endometrial cancers account for 2–5% of all cases (Arora 2020). The fact that women with hereditary endometrial cancer have an increased predisposition to many non-gynecologic malignancies increases the significance of the identification and counseling of these women and their relatives. The

identification of genetic signatures in these women may allow an early diagnosis and will help clinicians implement necessary strategic measures and preventive steps to better manage their risk of cancer. It will also help in providing reproductive counseling to patients who wish to extend their families.

16.4.1 Commercially Available Panels

Many commercial multigene testing panels for germline DNA testing in patients with endometrial cancer have been launched. The endometrial cancer panel from GeneDx, a US-based company, includes a list of 12 genes that are categorized into three main groups: high-risk, moderate-risk, and newer-risk. The high-risk genes include *BRCA1, BRCA2, EPCAM*, *MLH1, MSH2, MSH6, MUTYH, PMS2, PTEN,* and *TP53*. The moderate-risk genes include *CHECK2,* and the newer-risk genes include *POLD1*.

The most commonly used "PapSEEK Test" is an NGS-based whole exome detection for genes specific to ovarian and endometrial carcinoma, including *PTEN, PIK3CA, KRAS, FBXW7, CTNNB1, BRAF,* and *TP53*, from liquid pap smears of endometrial cancer patients. It is done to identify endometrial cancer at an early stage, even before symptoms are observed and cells are available for biopsy (Kinde et al. 2013).

Another NGS panel targeting gynecological malignancies, consisting of 156 oncogenes and tumor suppressor genes, known as the Einstein Custom Cancer Panel (ECCP), was developed to investigate somatic and germline genomic changes in various cancers (Miller et al. 2017).

Similarly, a comprehensive panel of endometrial cancer from Fulgent Genetics, which is also a US-based company, examines 11 genes associated with an increased risk of hereditary endometrial cancer. This includes *BRCA1, BRCA2, EPCAM, MLH1, MSH2, MSH6, MUTYH, PMS2, POLD1, PTEN, and TP53* genes. The *CHECK2* gene is not included in this panel.

Likewise, another US-based company, Ambry Genetics, launched the GYNplus panel, which provides a next-generation sequencing panel that simultaneously investigates 13 high-risk and moderate-risk ovarian and uterine cancer susceptibility genes. This panel includes: *BRCA1, BRCA2, BRIP1, EPCAM, MLH1, MSH2, MSH6, PALB2, PMS2, PTEN, RAD51C, RAD51D,* and *TP53 genes.* This panel includes four additional genes that are currently annotated for ovarian cancer risk only. These are *PALB2, BRIP1, RAD51C,* and *RAD51D*.

Some other nonspecific panels that assess the risk of hereditary cancers, including endometrial cancer, have made it to the market. This list includes the Myriad myRisk® Hereditary Cancer Test, a 35-gene panel that ascertains an elevated risk of eight hereditary cancers, including endometrial cancer. For endometrial cancer, it screens eight genes: *MLH1, MSH2, MSH6, EPCAM, PMS2, TP53, PTEN,* and *STK11*. The next in this list is "ColorGenomics," which includes *MLH1, MSH2, MSH6, EPCAM, PMS2, TP53, PTEN,* and *STK11* genes for endometrial cancer.

Thus, Myriad myRisk® and ColorGenomics have annotated the screening of STK11 as an additional gene pertinent to endometrial cancer risk.

NGS TruSeq Custom Amplicon Low Input from Illumina is an NGS-based panel designed for compatibility with blood samples, cell culture, fresh frozen tissue, etc. It is a targeted comprehensive cancer detection panel giving exon coverage of 409 cancer-associated genes and has been validated to detect variants within multiple cancer types such as lung, colon, breast, ovarian, melanoma, and prostrate malignancies. This panel has been used for prognosis in endometrial cancer patients (López-Reig et al. 2019). A more focused approach toward the identification of endometrial biomarkers is needed to develop more panels.

Recently, a very interesting and informative study evaluated the association of variants in 32 genes with the risk of endometrial cancer. The study concluded that out of these 32 genes, only six, namely *MLH1*, *MSH2*, *MSH6*, *PMS2, EPCAM,* and *PTEN,* can be considered suitable for clinical diagnostic testing of patients with endometrial cancer. The remaining genes can be subjected to further functional studies.

16.4.2 Genetic Classification of Endometrial Carcinoma

Several studies have identified various genes and pathways that are altered in the EECs (Lv et al. 2019). An integrated sequencing analysis of endometrioid carcinomas by The Cancer Genome Atlas (TCGA) in 2013 identified 48 genes with different mutation frequencies across the four groups (Xu et al. 2004; Levine and Network 2013). With the help of whole exome massive parallel sequencing, a mutation spectrum was utilized to construct a classification of endometrial cancer into four categories: POLE-ultra mutated, microsatellite instability (MSI)-hypermutated, copy number low, and copy number high. The POLE ultramutated group shows unusually high mutation rates and hotspot mutations in the POLE (Polymerase Epsilon) gene. The second subtype, MSI hypermutated, signifies high mutation rates and few copy number alterations. The low copy number subtype corresponds to the P53 wild-type group and corresponds to 2.9×10^6 mutations/Mb. The last subtype is copy number high, which exhibits 2.3×10^6 mutations/Mb (Bianco et al. 2020) and comprises widespread genomic alterations with extensive copy number alterations and P53 abnormalities.

16.4.2.1 POLE Ultramutated

This gene encodes the catalytic subunit of the DNA polymerase epsilon and is involved in DNA repair and chromosomal DNA replication. Somatic mutations in the exonuclease (proofreading) domain (EDM) of POLE are associated with genomic instability in endometroid carcinoma (León-Castillo et al. 2020). These pathogenic somatic mutations in EDM of the POLE gene define an important subtype of ultramutated tumors ("POLE-ultramutated") with ultramutation (frequency ≥ 100 mutations/Mb) (León-Castillo et al. 2020). These are present in 6–9% of cases of

EC. Somatic passenger mutations occur in PTEN, PIK3CA, TTN, etc., making the POLE mutation known as an ultramutation.

16.4.2.2 Microsatellite Instability

Microsatellite instability (MSI) has emerged as one of the crucial pathways in endometrial carcinogenesis (McMeekin et al. 2016). There are two possible causes that result in high microsatellite instability (MSI-high) in the tumor cells: one being a germline or sporadic mutation in at least one of the DNA mismatch repair enzymes, namely MLH1, PMS2, MSH2, and MSH6, and the other being epigenetic silencing due to promoter hypermethylation in the *MLH1* gene. MSI is predominantly reported in EECs; however, nonendometrioid endometrial cancers usually show genetic instability at the chromosomal level due to defects in the *TP53* gene. Lynch syndrome, also known as hereditary nonpolyposis colon cancer (HNPCC), is a cancer that is inherited in an autosomal dominant fashion. It is caused by mutations in genes of the DNA mismatch repair (MMR) repair system: MLH-1, MSH-2, MSH-6, or PMS2. Women with Lynch syndrome have an increased predisposition to endometrial cancer, and interestingly, *MLH1* mutations are seen in 24–40% of cases, and MSH6 mutations are seen in 10–13% of cases (Bonadona et al. 2011).

Additionally, Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*) is a tumor suppressor gene that negatively regulates cell cycle progression. Immunostaining of CDKN2A showed a striking overexpression in tumor cells of the serous ECs (Alkushi et al. 2010; Netzer et al. 2011; Reid-Nicholson et al. 2006; Yemelyanova et al. 2009). The endometrioid tumors, on the other hand, show very weak focal staining. Mutations in the *CDKN2A* gene are reported in 10%–28% of the EECs in comparison with 44% of the NEECs, showing that this gene is important in distinguishing serous carcinoma from endometrioid EC (Nakashima et al. 1999; Semczuk et al. 2003; Salvesen et al. 2005).

16.4.2.3 Copy Number High

This subgroup is characterized by genomic changes such as global amplifications and deletions, resulting in a serous tumor phenotype, particularly referred to as copy number high subgroup. TP53 mutations are common (more than 90% in type 2 and 11% in type 1 carcinomas) (Singh et al. 2020), and PTEN mutations have been observed at low frequencies in these patients. Some specific mutations occur in the FBXW7 (Cuevas et al. 2019), PPP2R1A, and PIK3CA genes in these cases (Cherniack et al. 2017). It belongs to serous or high-grade endometrioid tumors and hence, must be treated cautiously after surgery.

16.4.2.4 Copy Number Low

This subtype is characterized by tumors with amplification of chromosome arm 1q and CTNNB1 mutations (Depreeuw et al. 2017). About 30–60% cases show this subtype of EC. It is also known as the 'no specific molecular profile' (NSMP) subgroup. This type features microsatellite-stable grade 1 and 2 tumors with the lowest rate of mutations, commonly seen in the PTEN, CTNNB1, KRAS, and ARID1A genes (Levine and Network 2013). This group shows a response to

Table 16.1 Gene alterations specific to each subgroup of endometrial carcinoma genetic classification

Genes frequently altered	Genes alteration frequency in POLE-Ultramutated	Genes alteration frequency in MSI hypermutated	Genes alteration frequency in low copy number	Genes alteration frequency in high copy number
PTEN	94%	88%	77%	
PIK3CA	71%	54%	53%	47%
ARID1A	76%	76%	42%	
KRAS	53%	35%		
PIK3R1	65%	40%	33%	
FBXW7	82%			
ARID5B	47%	47%		
TP53				92%
PPP2R1A				22%

hormone therapy and possesses high progesterone receptor activity. The candidate marker genes belonging to these groups are mentioned in Table 16.1.

16.5 Alterations in the Epigenome

Though the epigenome has been demonstrated to have vital significance in endometrial cancer development and/or progression, there are not enough studies to conclusively define the epigenetic changes. A study by Banno et al. (2006) investigated the promoter methylation status of five genes: hMLH1, APC, E-cadherin, RAR-β, and p16 (Banno et al. 2006). The results displayed aberrant CpG island hypermethylation in the hMLH1, APC, E-cadherin, and RAR-β genes (Banno et al. 2006). Some other genes that reportedly show altered methylation in endometrial cancer include *HOXA11* and *THBS2* (Whitcomb et al. 2003). Similarly, Dowdy et al. (2005) reported aberrant methylation in the paternally expressed gene 3 (PEG3) in gynecologic cancer cell lines (Dowdy et al. 2005). Furthermore, Chan et al. reported hypermethylation of π-Class glutathione *S*-transferase (*GSTP1*) promoter in endometrial carcinoma (Chan et al. 2005). Hypermethylation of promoters of the genes PTEN and MLH1 is linked with Type 1 EC. Global hypomethylation and genomic instability are induced in Type 2 ECs (Inoue et al. 2021). Promoters of genes RASSF1A and GSTP1 showed hypermethylation and correlated with patients with higher tumor grades and myometrial invasion (Fiolka et al. 2013). These studies suggest significant epigenetic alterations in endometrial carcinoma, but their diagnostic and prognostic values yet remain to be established.

16.6 miRNAs as Potential Biomarkers in Endometrial Carcinoma

miRNAs tend to target tumor suppressor oncogenes or may themselves act as oncogenes with upregulated expression. Upregulation of the miR-200 family members (miR-141, miR-200a, miR-200b, miR-200c, and miR-429) and miR-203, miR-205, and miR-210 has been reported in the EECs (Lee et al. 2011; Yanokura et al. 2015), whereas downregulation of miR-410, miR-221, miR-15b, miR-17-5p, miR-125a, miR-20a, miR-214, miR-222, and miR-424 has been detected (Jia et al. 2013). These interpretations might prove influential in the identification of biomarkers that can assist in the noninvasive diagnosis of endometrial carcinoma. However, as with the methylation changes, the diagnostic and prognostic significance of miRNAs remains to be established.

16.7 Conclusions and Future Perspective

Endometrial carcinoma has a significant genetic component. Genetic investigations of these patients have identified mutations in a number of genes. Various combinations of these genes are commercially available in the form of genetic testing panels. These panels may be used in the diagnosis, prognosis, and treatment of EC patients with or without histological assessment of the endometrial tissue. The patients may ultimately be classified into groups, as suggested by the TCGA. Apart from helping the patients understand and treat the disease, genetic counselling for these patients may help manage the disease's transmission. In the case of individuals at risk of EC, these genetic testing panels may help in estimating the level of risk. Genetic testing in individuals with a family history of EC may help in taking preventive measures early in life. While genetic testing panels are available, further research would identify more genes that are suitable for adding to these panels, making them more informative. Apart from specific EC genetic testing panels, general cancer panels may also be used for testing, but only when necessary. In addition to genetic testing panels available today, epigenetic and small RNA-based panels will become available in the future. These markers may provide complementary information for use in genetic diagnosis, prognosis, and assessing the EC risk in individuals with a family history, budding the hope of treatment.

Additional studies for POLE sequencing and P53 immunohistology are being conducted to deduce a more specific panel. Targeted deep sequencing using a combination of NGS and clinical assessments is encouraged to decipher the role of ARID1A. Across many studies, we found TTK, CDC25A, and ESPL1 as potential biomarkers for endometrial carcinoma. CTNNB1 and FGFR2 mutations have been previously reported to have prognostic potential.

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Genetic Testing in Cervical Cancer

17

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Abstract

Cervical cancer, a prevalent disease in developing countries, is responsible for a huge number of deaths due to gynecological cancers. Human papillomavirus (HPV) infection is one of the major factors responsible for most cases. Genetic factors that define the interactions between the host and the pathogen, ultimately deciding the capability of the pathogen to cause cancer and that of the host to defend itself, are yet to be elucidated. There have been numerous screening methods that do not suffice for all diagnostic purposes or appear ineffective in reducing mortality. Due to the high mortality rate in women with cervical cancer, it is essential to identify important host-susceptibility factors. Genomic alterations, including changes in DNA, epigenome, and gene expression, can prove critical in identifying the underlying factors responsible for increased susceptibility to cervical cancer and its progression to severe forms. Genetic testing may have the advantage of identifying prevalent genetic mutations in a particular population. It might also benefit patients by providing ways to personalised medicine in the future.

Keywords

Cervical cancer · Genetic testing · Human papillomavirus (HPV) · Genetic susceptibility · Reproductive medicine

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17.1 Introduction

Cervical cancer is the fourth most common gynecological cancer worldwide, distinguished by high mortality rates, namely 25% in developing countries and 85% in poorly developed countries (Zhang et al. 2020). Infection with human papillomaviruses (HPV) is responsible for more than 95% of cervical cancer cases (Ferlay et al. 2015). Other responsible factors that account for 5% of cases include poor economic status, inadequate sexual and personal hygiene, smoking, sexual activity at an early stage, and having multiple sexual partners. It occurs in the cellular lining of the cervix. Almost all cervical cancers are either squamous cell carcinoma or adenocarcinoma (Rodríguez et al. 2008). Until now, 207 HPV genotypes have been classified based on their sequence differences in the highly conserved L1 gene region by at least 10% (Van Doorslaer et al. 2017). Cervical carcinoma is often due to HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (Walboomers et al. 1999). HPV type 16 holds the highest infection rate, and second place is given to type 18. The nononcogenic types include HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108. Bernard (2005) proposed the classification of human papillomaviruses, which have five genera: alpha, beta, gamma, mu, and nu (Bernard 2005). Alpha-papillomavirus is the most studied group of papillomaviruses since it is responsible for 5% of cancer occurrences worldwide. They are further categorized into low-risk and high-risk types based on their association with precancerous lesions or cervical cancer and into cutaneous or mucosal types based on their ability to infect the epithelial skin cells or inner tissue lining.

Induction of cancer takes a series of molecular events such as viral transmission, persistence, and progression to form precancer and invasion. These events are not yet fully understood. Dysplasticity is an integral characteristic of cervical intraepithelial neoplasia (CIN) and cancer. Low-grade CIN shows mild dysplasia, which is characterized by dysplasia in the lower one-third of the epithelium (Anderson et al. 1991). In high-grade CIN (CIN2 and CIN3), CIN2 shows dysplastic growth in two-thirds of the epithelium, and CIN3 is severe dysplasia that affects the full two-third thickness of the epithelium (Arends et al. 1998). High-grade CIN is a severe form of cervical cancer, which should be clinically managed as soon as it is detected.

17.2 Pathogen Genome

The 8-Kb genome of HPV possesses three functional regions: an early region (E), a late region (L), and a long control region (LCR), also called upstream regulatory region (URR). The early region (E) includes E1, E2, E4, E5, E6, and E7 genes. The highest variation in the viral genome expression is due to the LCR region. If the E2 protein is lost, the transcription of E6 and E7 is upregulated. The loss of E2 protein acts as a hallmark of the initial establishment of cervical cancer. E4 protein doesn't have transforming activity yet but plays a role in the later stages of infection. E6 inactivates p53, and E7 degrades retinoblastoma tumor suppression protein (pRB)

and affects the mechanisms of apoptosis and cell cycle arrest to induce cancer in humans. It leads to genetic mutations in the infected cells. E7 disrupts the functional association between pRb and HDAC1, leading to squamous cell carcinoma. The late region, comprising L1 and L2 ORFs, encodes for major and minor capsid proteins, respectively. The URR region is composed of numerous binding sites for viral E1 and E2, which are essential for HPV replication initiation. Along with that, it includes NFI, Oct-1, AP-1, TEF-1, and SP1 for transcription initiation. It implies that it plays a significant role in identifying a variety of hosts for specific HPV types.

With the progression from the primary stages of lesion development toward cancer, there is overexpression of E6 and E7 at the full-thickness epithelial lesion stage. The differentiated HPV-infected squamous epithelial cells have the capability to replicate HPV particles and express L1 or E4 proteins. It can be inferred that L1 or E4 represents multiplication of the virus epigenome and can be used as a marker. The DNA of the virus is located extrachromosomally in the benign lesions and gets integrated with the host genome in high-grade intraepithelial neoplasia and invasive cancers. The virus integration into the host genes and regulatory elements causes the host genome to exhibit structural changes and show transcriptional dysregulation. Carcinogenesis is marked by TLRs regulation that elicits an antiviral response via the IFN-regulatory factor (DeCarlo et al. 2011). Some studies concluded that TLR-9 acts as a marker during cancer development due to its evident lower expression in CIN1 than high-grade cervical intraepithelial neoplasia (CIN) and highest expression in squamous cell carcinoma (Ghosh et al. 2015). E6 and E7, besides their role in apoptosis, also function to downregulate TLR-9, leading to immune response evasion and ultimately infection persistence. Modulation in E7 can alter TLR9 signaling.

17.3 Targeting the Pathogen

Testing for pathogen presence requires the detection of E6-E7 proteins using ELISA for instant results, which has been in practice for a long time, but lacks sensitivity. If HPV screening is to be done with 100% sensitivity and specificity, genetic changes must be elucidated and gene panels must be developed. The technique of restriction site polymerase chain reaction (RS-PCR) detects the sites of HPV integration. It tells about the common fragile sites with sizes varying from 100 Kb to 9 Mb, which have a role in deletions, translocations, and even the events of gene amplification common in tumor cells (Wenger 1995).

FRA3B (3p14.2), located within the fragile chromosomal region, gets altered in cervical cancer. 1.5-Mb FHIT gene that lies in the center of FRA3B provides a site for HPV integration within its intron 3. Similarly, 23 HPV16 integration sites were found by the Thorland team in 2002 (Thorland et al. 2003). Identification of these sites could help shed light on determining the host–pathogen interaction and help find the susceptibility to cancer development in the host. Next-generation sequencing has revealed that the genes encoding the human leukocyte antigen system (HLA) are responsible for the increased susceptibility to cancer.

Methylation studies have revealed that the HPV16, HPV18, HPV31, HPV33, and HPV45 genes get methylated during carcinogenesis. HPV16 L1 and L2 ORFs showed an association between increases in methylation levels and the onset of CIN2, CIN3, and invasive cancer. Snellenberg et al. (2012) showed that methylation of several HPV-16 E2 binding sites is significantly higher in invasive cervical cancer than in situ cervical lesions (Chaiwongkot et al. 2013; Snellenberg et al. 2012). HPV1 genomes extracted from warts had high levels of methylation in the viral late regions and low levels of methylation in the upstream regulatory and early regions, which were similar to the methylation patterns seen in the genital high-risk HPV types. Lippert et al. (2021) constructed an NGS panel, targeting seven regions of the HPV-16 viral genome and two viral regions for the remaining 26 HPV types (Lippert et al. 2021). This study established the need for NGS-based HPV detection tests that may identify several oncogenic HPV variants. Pioneering platforms such as Illumina (CA, USA) and Thermo Fisher Scientific (MA, USA) allow high-throughput and cost-effective sequencing.

17.4 Host Genome and Potential Targets

Humans as hosts welcome pathogens with a sword of immune cells. However, pathogens have similarly evolved mechanisms to evade the host immune system. HPV entrance into the body decreases cytokine production and suppresses NF-κB by triggering EGFR upregulation (Nees et al. 2001). This leads to a series of proinflammatory events and promotes inflammation. Upregulation in the levels of Tregs, IL-10, and TGF- β enhances the transformation of normal cells into cancerous lesions. TGF-β1 increases in lesions progressing from CIN-1 to invasive cervical cancer and hence can be of prognostic value (Molling et al. 2007). Similarly, immune system IL receptor 1 IFNAR1, interleukin 1 receptor antagonist (IL1RN) genes, and epithelial membrane protein 1 (EMP1) are also altered (Gius et al. 2007). The proportion of tumor-infiltrating CD8 T cells to Foxp3 T-regulatory (Treg) cells acts as a significant independent prognostic factor in cervical carcinoma. Host–pathogen interactions suggest that tumor-activating genes occupy a special space in the consortium of potential biomarkers. The E6-AP complex of the pathogen targets the P53 gene in the host via E6AP ubiquitin ligase. Similarly, cross talk of P53 with the CDK inhibitor family, majorly with CDKN1A, suggests that single-nucleotide polymorphisms in the CDKN1A codon Arg31Ser can be associated with cervical cancer (Roh et al. 2010).

Another member, CDKN3 mRNA upregulation, denotes tumor progression as well as survival possibilities for the patient. We can also conclude that the CDKN3 gene could be a good therapeutic target for tumor growth inhibition (Barrón et al. 2015). Bioinformatics studies along with molecular study validation by RT-PCR and IHC have confirmed the potential of mitosis-associated proteins, such as NUSAP1 and CDC20, along with CDKN2A, in differentiating high-grade CIN from low-grade CIN (Espinosa et al. 2013). p16INK4a is an important CDK inhibitor that acts as a prominent signature marker of deregulated HPV oncogene expression.

Many protein-based tests have been developed to detect p16INK4a that can be introduced as point-of-care tests for cervical cancer diagnostics, yet they are not in practice because of high labor and cost needs.

Many polymorphisms have been reported to be associated with cancer risk. For example, novel mutations in SHKBP1, ERBB3, CASP8, HLA-A, and TGFBR2 have significance in the occurrence of cervical cancer. Other genes linked with poor cervical cancer patient survival are LIM kinase 1 (Chhavi et al. 2010), galectin 7 (Tsai et al. 2013), NF-kB (Wu et al. 2013), and caspase-3 (Hu et al. 2014). Invasive cervical carcinomas invariably demonstrate a gain of the human telomerase gene (TERC) due to extra copies of chromosome arm 3q (Horng et al. 2004). MICA (MHC class I chain related gene A), expressed by keratinocytes and epithelial cells, interacts with γ-δ T cells and influences the pathogenesis of cancer by presenting viral or tumor antigens. Ghaderi et al. (1999) and Hanbyoul Cho (2014) showed MICA to be a potential marker for displaying susceptibility to squamous cell carcinoma

17.5 The Provision of Genetic Testing

Despite regular human papillomavirus (HPV) testing and detection by cytology, visual inspection with acetic acid (VIA), and visual inspection with Lugol's iodine, which are known as standard screening tests for cervical cancer, still 98% of cervical cancer cases are reported to arise due to the scarcity of reliable screening tools. No reliable screening methods are available so far.

Due to their critical involvement in the etiology and control of cancer, HPVs must be identified for accurate diagnosis and subsequent treatment. The hc2 clinical test, which targets 13 oncogenic HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) without discriminating between them all, is frequently used to detect HPV. However, merely detecting the HPV presence is not sufficient; identification of the HPV type is critical in understanding the risk. Three commercial platforms (Digene Hybrid Capture 2, Qiagen, Germantown, MD, USA; Cobas, Roche Diagnostics, Indianapolis, IN, USA; and APTIMA, Hologic, Marlborough, MA, USA) have been approved by the U.S. Food and Drug Administration (US-FDA) for the detection of high-risk HPV in liquid cytology specimens, but they are not sufficient to provide HPV genotype with accuracy. DNA-DNA hybridization methods and restriction endonuclease cleavage patterns have made it simpler to detect HPVs in cervical biopsies and exfoliated cells. Quantitative polymerase chain reaction (qPCR) technology has exploited a range of primer sets for amplifying various sections of the HPV genome. The MY09/MY11 primer set and the GP5+/GP6+ primer set both target the conserved L1 region of the HPV DNA in clinical samples. A novel consensus primer specific for the E1 HPV gene is used in the PCR-based PapilloCheck test. Other PCR-based identification involves the utilization of various consensus or degenerated primers (broad-range primers). These primers usually detect recognized HPV types but might also detect some potentially novel types. PV primers such as ARL1(E1), FAP6085/FAP6319,

FAP59/FAP64, CODEHOP, CUT, CPI/CPIIg (s), Ma/Ha, and a set of CP and CN primers target L1 and E1 conserved regions of the HPV genome. Rolling circle amplification is another intriguing technique for finding unique nucleotide sequences. It can be utilized for more targeted amplification of HPV genomes due to its high fidelity and large amplicon size.

Due to the labor-intensive nature of genotyping assays, NGS-based HPV genotyping has been introduced for formalin-fixed paraffin-embedded (FFPE) samples that provide more sensitive outcomes. As discussed above, HPV CpG site methylation levels are well correlated with high-grade cervical lesions in many studies, and these can be determined using the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) method. However, NGS assays yield sufficient information on single-molecule CpG methylation levels that can help reveal the extent of methylation in cervical cancer. Table 17.1 summarizes the studies demonstrating the contribution of NGS to better investigate and classify cervical cancer stages.

17.6 Cancer Susceptibility Determining Genes

Genetic susceptibility corresponds to disruptions in apoptotic and cell immune signaling pathways. It is claimed that alterations in gene levels result in familial aggregation, thus showing prolonged cancer history. Genes responsible for cell growth, proliferation, migration, and death are majorly mutated when cancer progresses from mild to high-grade stages. In total, 85% of invasive cervical carcinomas have shown a copy number increase in chromosome arm 3q. Copy number variations in the PIK3CA, BRCA1, BRCA2, ATM, and TP53 gene are well known. The Cancer Genome Atlas Project (CGAP) reported the association of mutations in SHKBP1, ERBB3, CASP8, HLA-A, and TGFBR2 genes with a higher risk of cervical cancer. More than 10 sequencing studies have reported genes, such as PIK3CA, MTOR, KRAS, MAPK1, and HLA-B, responsible for CC progression, which were further validated by the KEGG and ONCOKB databases. GWAS studies described that genetic susceptibility variants, including PAX8 (rs10175462), CLPTM1L (rs27069), HLA-DQA1 (rs9272050), MICA (rs6938453), HLA-DQB1 (rs55986091), and HLA-B (rs92666183), are found to be linked with CIN3 and invasive cervical cancer that indicate disruptions in apoptotic and immune function pathways (Qiu et al. 2022) and can act as probable biomarkers for cancer inheritability. p21 Codon 31 with the AGA allele exhibits a genetic risk factor for cervical SCC (Tian et al. 2009). According to CGAP, 70% of the cervical neoplasia showed aberrant genomic alterations in the PI3K/MAPK and TGF-beta signaling pathways, because of which its genetics resembled that of endometrial cancers. These endometrial-like cancers are HPV-negative and exhibit high frequencies of mutations in the ARID1A, KRAS, and PTEN genes. Other genes such as BCAR4, CD274, and PDCD1LG2 can be used as therapeutic targets. Studies have also reported that the upregulation of Survivin and FasL and the downregulation of Fas might be responsible for cervical carcinogenesis (Wu et al. 2012). SNP analysis at

Table 17.1 Sequencing-based detection of stages in cervical cancer using different specimens

Sr no.	Techniques for detection	Sample used	Outcome of investigation	References
1	MS-restriction digestion and bisulfite sequencing	FFPE biopsy samples	NILM, CIN1–3. SCC	Badal et al. (2003)
2	MS-restriction digestion	Fresh frozen biopsies	NILM, CIN1-CIN3, ICC	Hublarova et al. (2009)
3	Pyrosequencing	Exfoliated cervical cells	≤CIN1, CIN2+	Piyathilake et al. (2011)
4	Bisulfite sequencing	Exfoliated cervical cells	< CIN2/3, CIN2/3	Xi et al. (2011)
5	MS-restriction digestion	Fresh frozen biopsies	CIN1–3, ICC	Mazumder Indra et al. (2011)
6	MS-restriction digestion and bisulfite sequencing	Fresh frozen biopsies	NILM, SCC	Bhattacharjee and Sengupta (2006)
7	Pyrosequencing	Exfoliated cervical cells	NILM, CIN1–3, ICC	Hong et al. (2008)
8	Bisulfite sequencing	Cervical scrapes/biopsy tissues	LSIL, HSIL, SCC	Ding et al. (2009)
9	LCM, bisulfite sequencing, COBRA	FFPE tissue sections	NILM, LSIL, HSIL	Vinokurova and von Knebel Doeberitz (2011)
10	Methylation independent PCR, Luminex xMap	FFPE biopsy samples	NILM, CIN3, SCC	Snellenberg et al. (2012)
11	Bisulfite sequencing	Cervical cells	NILM, ASC-US, LSIL, CIN1–3	Brandsma et al. (2009)
12	LCM, bisulfite sequencing	FFPE biopsy samples	NILM, ICC	Kalantari et al. (2009)
13	Bisulfite sequencing	Exfoliated cervical cells	LSIL, HSIL	Kalantari et al. (2010)
14	Pyrosequencing	Exfoliated cervical cells	NILM, LSIL/CIN1, CIN2/3, ICC	Sun et al. (2011)
15	Pyrosequencing	Exfoliated cervical cells	Cleared HPV, persistent HPV, CIN3	Mirabello et al. (2013)
16	Pyrosequencing	Exfoliated cervical cells	<CIN2/ASCUS, CIN3	Wentzensen et al. (2012)

various research strata identified the IFNG, TMC6, and TMC8 genes as being closely linked with cervical cancer development (Wang et al. 2010). These genes are involved in tumor immune surveillance and are responsible for innate as well as adaptive immunity.

17.7 Epigenetic Markers for Cancer Progression

For screening human papillomavirus (HPV) infection in women, nucleic acid methylation has been advocated as a one-step molecular diagnostic and prognostic test that may someday directly complement or replace HPV traditional screening methods, which lack sensitivity. Early detection of cervical cancer precursor lesions can be done by developing a panel of methylation markers. Genes such as DAPK1 (death-associated protein kinase 1) showing promoter hypermethylation can be an indication of the severity of cancer (Agodi et al. 2015). Bisulfite-pyrosequencing of genomic DNA showed promoter methylation of four candidate tumor suppressor genes, adenylate cyclase 8 (ADCY8), cadherin 8, type 2 (CDH8), MGMT, and zinc finger protein 582 (ZNF582), in cervical carcinomas as no exception (Shen-Gunther et al. 2020). A panel for assessing high-risk HPV positive candidates using methylation-specific PCR panels targeting the CADM1/MAL genes can be developed to substantiate the screening tools for better diagnosis. Methylation studies have confirmed the CCNA1 gene as a potential biomarker that can differentiate CIN3 from CIN2 (Binnicker et al. 2014). Hypermethylation of SOX1, TERT, hsa-miR-124, and LMX1A is correlated with CIN 2 (Rogeri et al. 2018). Methylation pattern in both EPB41L3 and JAM3 genes is a precise and practical screening method for CIN2 (Kong et al. 2020). Some signature biomarker panels consisting of CXCL13, TP63, and DSG3 can differentiate between CIN1 and carcinoma based on methylation library sequencing outcomes. The availability of such validated and potential biomarkers can enhance the development of new epigenetic therapeutic regimes.

17.8 Emerging Biomarker: circRNAs

circRNAs, the noncoding endogenous subtype of RNA without the 5′ end cap structure and 3′ end polyA tail structure, are evolutionarily conserved. By serving many roles, such as targeting mRNAs, forming RNA–protein complexes, and regulating gene transcription as well as translation machinery, circRNAs also regulate signaling pathways associated with cell growth, proliferation, and apoptosis. As tumor cells exhibit DNA methylation, histone modifications, these circRNAs have the potential to be used as biomarkers of cervical cancer (Chen et al. 2021). A specific circRNAs identification panel (Arraystar Human circRNA Array Technology) was designed with the assessment based on differentially expressed circRNAs between one normal cervical epithelial cell line and five cervical cancer cell lines. Many circRNAs have been checked for their functions, targets, and respective roles in cervical cancer progression, as mentioned in Table 17.2 (adopted from the review article by Shuying Chen et al. 2021). Upregulations of circRNA-000284 and circRNA-102,049 and downregulations of circRNA-001622 and circRNA-104,892 were observed (Holdt et al. 2018). This assay can be further challenged and utilized for finding the relation between circRNA and the occurrence and development of

Table 17.2 circRNAs with their unusual expression denoted with function and clinical relevance in cervical cancer (modified from Chen et al. 2021)

CircRNA	Change in level	Target genes	Function	Clinical relevance	References
circRNA_0000263	Up	miR-150-5p	Proliferation and migration	Tumor size and lymph node metastasis	Cai et al. (2019)
circRNA_0000745	Up	E-cadherin	Proliferation, invasion, and migration	Tumor differentiation and lymphatic invasion	Jiao et al. (2020b), Zhang et al. (2020)
circRNA_0007534	Up	miR-498	Proliferation and invasion	Tumor size	Lopez-Serra and Esteller (2012)
circRNA_0031288	Up	Hsa-miR-139-3p	Invasion and migration	Lymph node metastasis	Song et al. (2019)
circRNA_SLC26A4	Up	miR-1287-5p	Proliferation and invasion	Tumor size	Ma et al. (2018)
circRNA_AKT1	Up	miR-942-5p	Proliferation and invasion	Tumor size	Zhou et al. (2019)
circRNA_CLK3	Up	miR-320a	Proliferation, invasion, and migration	Tumor differentiation, FIGO staging, and stromal infiltration	Feng et al. (2019)
circRNA_8924	Up	miR-518d-5p	Proliferation, invasion, and migration	Tumor size, FIGO staging, and muscle invasion	Sun et al. (2019)
circRNA_0000285	Up	miR197-3p-ELK1	Cycle and apoptosis	Tumor size	Yu et al. (2018)
circRNA_EIF4G2	Up	miR-218	Proliferation, invasion, and migration	Tumor size and lymph node metastasis	Mao et al. (2019)
circRNA_0084927	Up	miR-142-3p	Proliferation, invasion, migration, and cycle	Tumor size	Chen et al. (2020)
circRNA_FoxO3a	Up	–	Infiltration and migration	Stroma infiltration and lymph node metastasis	Tang et al. (2020), Yang et al. (2021)
circRNA_101308	Up	miR-26a-5p	Proliferation, invasion, and migration	Deep muscle layer infiltration and lymph node metastasis	Jiao et al. (2020a)

cervical cancer through mutually associated oncogene-related mRNAs and miRNAs.

17.9 Conclusions and Future Perspective

Molecular analysis by HPV-DNA detection is crucial despite the availability of prognostic factors including FIGO stage identification, lymph node status, and calculation of tumor size, depth, histology, lymph-vascular space involvement, and diffusion in the uterine body. However, the processes of infection, pathogen invasion, integration in the host, immune reaction, and the development of carcinoma are complex phenomena that must be disentangled before molecular approaches can be fully utilized. It is crucial to understand the mechanisms underlying the variables mediating signaling disruption, pathway differentiation, and alterations in epigenetic chromatin dynamics. While there are methods to identify the presence of HPV, further characterization is critical to understanding the course of the disease and its treatment. The role of NGS in detecting and identifying the subtypes of HPV is unparalleled and would serve multiple purposes to help in the management of cervical cancer. Further research on host and pathogen genetics would help identify important host-susceptibility factors linked to invasive and metastatic cervical cancer. Other tools, such as the development of epigenome and small RNA-based markers would take time, as a lot needs to be unraveled before they can be exploited for the detection or prognosis of cervical cancer. It is not only the pathogen genetics that needs attention, but the host susceptibility factors also need to be delineated. It is possible that NGS-based detection of HPV and its types will become critical to the management of cervical cancer in the future. The expansion of our knowledge regarding the relevance of HPV subtypes/variants and the genetic aberrations in the virus and host genome can contribute heavily to the management of cervical cancer. The availability of multiple molecular markers may help in the better management and prognosis of cervical cancer.

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Ethical Solicitude in Reproductive Carrier Screening

18

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Abstract

The reproductive carrier screening that helps people know their own carrier status sometimes prevents an individual from having children or take their own reproductive decisions. Therefore, reproductive carrier screening raises a lot of ethical issues, such as justice, consequences, equity, and autonomy, which turn out to be a cautious step during any screening program design or implementation. Ethical issues detailed in this chapter are mainly concerned with highlighting issues like embryo selection, abortion, interfering with societal norms, reproductive autonomy, informed consent, population-specific variants, and modifying genetic makeup. We should look up to such a screening program that is designed to keep ethical issues in consideration so that it does not breach human sentiments or create any kind of ethical pressure, which may also result in its easy adoption.

Keywords

Genetic testing ethics · Ethical issues in carrier screening · Carrier status · Genetic medicine · Reproductive medicine · Reproductive genetic testing

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18.1 Introduction

Reproductive carrier screening, which helps in providing large-scale information on the possibility or risk of disease transfer to the upcoming generation, brings along a number of challenges for its adoption, be it at the level of counseling, awareness, reproductive choices, stigmatization, technical knowledge, etc. Among many of the challenges that reproductive carrier screening faces, the major one is ethical issues. Ethical issues and overall screening program implementation are deeply rooted together and dependent on each other. Ethical issues often interfere with the main objectives of the screening, which makes it difficult to implement population-wide screening for a healthy future (De Wert et al. 2012). Broader implementation and the inescapable comprehensive aspect of reproductive carrier screening somewhat tend to also increase ethical concerns. Ethical concern also arises due to the unacceptable goals of screening programs set by society, including ignoring recipient consent of choice, faulty result reporting, and breaching community values (Kihlbom 2016). Some populations also face forced screening for a particular disease, making them indifferent to the rest of the population of the world, making it an important issue that needs to be checked (Dive and Newson 2021). Therefore, while screening, it is very important to strike the right balance between the patient's privacy, the confidentiality of genetic information, and family interests to avoid many ethical issues. This is to be noted that all the ethical issues may eventually result in legal issues if action is taken by breaching laws. The ethical issue leads to the legal concern of discrimination, privacy legislation, and interfering with individual and family rights (Otlowski and Williamson 2003). The ethical issue is also a barrier to promoting carrier screening in reproductive health. In this chapter, we will discuss different ethical issues faced during screening or postscreening.

18.2 Ethical Issues During Genetic Counselling

Reproductive counseling at the level of genetic clinics helps enhance opportunities for taking up reproductive choices. During counseling on available reproductive choices, one of the ethical issues is abortion or embryo selection (De Wert 1999; Knoppers et al. 2009). The abortion of a defective child creates an ethical concern due to the moral status of the human embryo and fetus. This ethical concern varies among individuals, as some of them find an embryo just a mass of cells and tissue with no moral status while others find it to have a moral status just equivalent to a person (https://www.healthcouncil.nl/documents/advisory-reports/2004/04/29/prenatal-screening-2-down's-syndrome-neural-tube-defects; Knoppers et al. 2006). In between these two extremes, some feel that the embryo and fetus are real but of low moral status, which can be neglected to avoid a genetically affected child. However, some people believe that the moral status of the embryo increases as it grows, which further increases ethical concerns about abortion (Boonin 2003). Preimplantation diagnosis and prenatal diagnosis are prohibited in countries like Germany due to ethical concerns (Finck et al. 2006). Counselors in countries like Saudi Arabia face

ethical issues with the termination of pregnancy due to religious beliefs (Balobaid et al. 2016).

Counseling generally tends to be both directive and nondirective. Directive counseling is where counselors tend to give reproductive choices to patients based on their own experience and anticipation (Wertz and Knoppers 2002). Nondirective counseling is where counselors do not make any judgment of their own, i.e., they avoid giving any reproductive choices to the patients and help them make their own decisions, which is known as reproductive autonomy (De Wert 1999). To avoid ethical issues to a certain extent, a counselor must be qualified enough to offer nondirective counseling to help couples at risk reach a reproductive decision. Generally, directive counseling should be avoided, as at some it hinders reproductive autonomy, but it should be offered when cases are too serious. Therefore, drawing a line between risky and nonrisky proportions must be done before giving directive counseling (Elwyn et al. 2000).

Yet another problem faced by counselors is preserving one's reproductive autonomy. Reproductive autonomy is defined as a person's ability to reflect on their values and goals while making a reproductive decision. The decision must be taken keeping in mind its long-term effect on values and the life they wish to live (Van der Hout et al. 2019). On the other hand, reproductive decisions are and must be taken by couples and should not be driven by societal norms, despite having genetic abnormalities. In order to avoid pressure on couples and pregnant women to undergo abnormality tests and terminate an affected pregnancy, the counselor's focus should be on enhancing a patient's reproductive autonomy along with avoiding a defective child, thereby respecting couple's values and preferences (Buchanan et al. 2001). However, it is quite impossible to consider both cases, i.e., reproductive autonomy and eliminating defective children, together as most of the screening programs consider eliminating defective children as their primary aim, thus creating ethical issues (De Wert et al. 2012). Counselors were more directed toward preventing defective childbirth, leading to ethical issues, until the 1980s when nondirective counseling came into effect, where reproductive autonomy is also considered (Kessler 1992). The ongoing ethical dilemma is whether to consider autonomy or public health. Counseling for reproductive autonomy should not be misinterpreted to lead to consequences like gender selection and aborting hearing children to deaf parents (Buchanan et al. 2001). Clarke said that counselors should not guide patients about any predetermined outcome; they should support patients to reach their decision through nondirective counseling (Clarke 2017).

The ethical problem is also about the lack of information or consent of the patients, whether screening prenatally or preconceptionally (De Jong et al. 2010). To work on this ethical issue, counselors should give patients all the necessary pre-test information, specifically the implications of the possible outcome. Whereas, counselors should help in providing sufficient pre-test information like the aim of the test, procedure, and implications of the possible outcome. This is just to work on the notion of informed choices and emphasize autonomous decision-making. Informed consent will help patients by knowing the pros and cons and taking better decisions without any psychological impact fitting into their values and beliefs (Marteau et al.

2001). When patients have accurate information, they are more likely to take sensible decisions. Sometimes information does not turn out to be beneficial or relevant and is confusing, which creates more issues. Therefore, it is possible to have a negative effect on decision-making by giving too much information. One should avoid a situation where ethical issues arise as patients make decisions and ask for screening because of prejudice, misconception, and pressure.

When screening is offered preconceptionally to school children and adolescents, it was ethically challenged to have a psychological impact and the feeling of not being normal if tested positive, but now this approach has been found to benefit by ensuring equity along with increased awareness in the population as a whole (Lakeman et al. 2008; Modra et al. 2010). According to a Jewish Orthodox community, the targeted group felt stigmatization combined with the loss of self-esteem; therefore, it was stated that it all depends on how screening is delivered and what information is presented before the test (Raz and Vizner 2008). The UK Human Genetics Commission emphasizes that adolescent screening can be acceptable only if it protects their reproductive autonomy (https://tegalsi.hypotheses.org/files/2011/04/2011.HGC_.-Increasing-options-informing-choice-final1.pdf).

Preconceptional screening or genetic screening creates ethical debate by discriminating against those who already have these faulty traits (Parens and Asch 2000; Shakespeare 2006; Janssens et al. 2014; Scully 2008). In order to avoid ethical issues in population-based reproductive screening programs, it must be designed in such a way that it does not create any stigmatization for the people living with such genetic conditions, and only genes associated with severe childhood-onset conditions should be considered (Savell and Karpin 2008).

Counselors face problems where ethical issues like cultural norms are hindered. It is quite obvious that people do not want to change their societal norms and cultural values, and taking any reproductive decision is quite tough. Consanguinity is one of the major problems for many genetic disorders, and counselors tend to create awareness about the demerits of getting married within families, but it hurts the cultural values of a population (Middle et al. 2007). Further ethical challenges arise when a woman is tested positive and experiences stigma from her husband and relatives, and hence, she hides such information from her daughter in fear of her future. Counselors also face problems in requesting the fiancé of a carrier female to be tested for screening (Lehmann et al. 2011). Thus, counselors face the problem of creating ethical issues of uninformed knowledge or consent from the fiancé and, at the same time, have to consider the confidentiality of carrier women's results (Balobaid et al. 2016).

18.3 Ethical Issues in Gene Selection

The screening was initially done in people and families with a history of inherited diseases, while such practices regardless of family history were not warranted until some people with no such history developed the disorder, and occasionally people who tested negative during screening developed a diseased condition in their

offspring. The ethical debate also includes the selection of variants that are to be targeted during the screening process (Kirk et al. 2021; Rowe and Wright 2020).

Uncertainty regarding the pathogenic nature of a genetic variant is an issue. Ethical concerns also stem from the fact that the likelihood of a variant being disease-causing in a population may be very different in comparison to another population. Thus, affirming a variant to be disease-causing and implementing screening in a population on that basis might result in unnecessary trouble for a population that is not actually susceptible. Other ethical concerns related to variants that are not reported as disease-causing variants due to low chance result in screening, which actually resulted in a defective child having a serious concern. Genes and variants to be selected for screening create ethical concerns as variant databases are being developed (Dive and Newson 2021; Wienke et al. 2014). While selecting a gene and variants to be included in the panel of screening, its severity, variable penetrance, expressivity, and scalability must be considered, which individually can give rise to ethical issues (Dive et al. 2022).

The severity of the condition connects with ethical issues arising out of the consideration of the inclusion of conditions in the screening program. Severity must be considered carefully; for example, a screening program conducted in Australia considered only those diseases in the panel, the confirmation of which led the carrier to choose to abort the child or avoid childbearing altogether, making such testing very critical (Dive et al. 2022; Kirk et al. 2021). Another example is when a population is considered to be screened and only life-threatening or severe conditions are targeted. While selecting a disease on the basis of its severity, the ethical and practical complexity that it will generate is also important. The selection of a severe condition to be included in the panel can be defined depending on whether we are selecting genes for families with existing conditions or screening without a history (Inthorn 2014; Thomas et al. 2020; Arjunan et al. 2020; Dive et al. 2022).

Some gene mutations show varying penetrance depending on a number of factors, such as genetic background, epigenetic changes, and environmental factors. Incomplete penetrance is the condition where a mutation associated with a disease is present but does not result in the disease phenotype. Varying penetrance leads to a disease only in some populations or a subset of people. Variations in expressivity give birth to uncertainty and make the implication of carrier screening very difficult, giving rise to ethical issues (Dive and Newson 2021; Gregg et al. 2021; Wright 2022). The ethical issue also arises while deciding the variants to be reported, i.e., whether it will be worse if screening does not detect a carrier individual with a high chance of a disease condition or reporting variants that are complex and of uncertain significance. Reporting career screening results irrespective of their penetrance makes people indecisive (Rowe and Wright 2020; Kirk et al. 2021; Dive et al. 2022).

When a screening program is scaled up to a population level, there are ethical issues that are completely dependent on how the design and implementation of the program have been done and how reporting and counseling are to be done (Dive et al. 2022). There are variants that turn out to be lethal when expressed in combination with other variants; however, the genetic background is not always

fixed, and this gives rise to ethical concerns when a counselor tends to explain it to the participants, affecting their reproductive decision-making (Gregg et al. 2021; Dive et al. 2022). Genetic variants and their impact on health are constantly evolving; therefore, the counselor must be updated regularly for the implementation of a population-based screening program. In conclusion, scaling up a screening program to the population level needs ethical considerations as most of the information might be ambiguous and uncertain at the population level.

18.4 Ethics and Eugenics

Eugenics is the practice of choosing desirable characteristics in a species by manipulating breeding and heredity. Screening programs that ultimately choose which types of people will be born and which types of people will be less desirable in the future may create ethical issues similar to eugenics. Reproductive screening is somewhat promoting eugenics, and as the panel for screening increases, it promotes more attributes of eugenics (Kirk et al. 2021; Mehler 1987; Stern 2002; Kevles 1995; Dive and Newson 2022; Galton 1883). Thus, it is important to differentiate reproductive carrier screening from eugenics and its ethical impact. What other than the Nazi regime in Germany can be more suitable to explain eugenics? Altogether, eugenics goes morally wrong, thus giving rise to ethical debate (Dive and Newson 2022; Galton 1883). The only difference that stands true between eugenics and reproductive carrier screening is that the former was introduced to improve the population genetic pool, while the latter is more inclined toward individual genetic makeup (Stern 2002; Dive and Newson 2022; Galton 1883; Robertson et al. 2019). Although reproductive carrier screening is also implemented in a population, extra care is to be taken so that it remains distinct from eugenics. The eugenics approach to reproductive carrier screening is discouraged as its practice discriminates against people with disabilities (Scully 2008; Shakespeare 1998; Parens and Asch 2003). When screening is offered preconceptionally, it has more ethical notions, which tend to select a "better fetus," falling in line with eugenics attributes (Daar 2017; Harwood 2020). Another way of distinguishing eugenics and carrier screening is to put more emphasis on a counseling session that would help the subjects reach a particular decision using their reproductive autonomy. However, RCS, when implemented in the entire population, somehow projects ethical issues that are common with eugenics. An increased panel of RCS that tends to alter upcoming genetic makeup sends a message that people having those genetic conditions are less desirable and valuable and some people will look inherently superior to others. Thus, the social impact of such programs must be checked (Kessler 1992; Dive and Newson 2022; Biesecker 2001).

The interpretation of society about disability and the perception of RCS do matter for an individual's reproductive decisions. People will bring different life experiences, values, family contexts, cultural factors, socioeconomic dimensions, and perspectives that will be a hurdle to the reproductive choices available to individuals. People do make decisions according to societal norms and contribute

to the future genetic pool accordingly; thus, it is not possible to escape eugenic attributes just by emphasizing freedom of individual reproductive choices. Along with reproductive autonomy, genetic variants, and their knowledge should be socially informed, i.e., understanding of what it would be like to live with different kinds of abilities. Deformities are not limited to physical or intellectual impairments, but to some extent, also depend on social and environmental responses to the disability. Thus, understanding disability will help to promote RCS in a better and more positive way with fewer ethical interventions like societal acceptance, support for people with disabilities, and a decreased disability-prone future (Dive and Newson 2022; Scully 2018; Rubeis and Steger 2019). Government-funded RCS that are used on the entire population face the risk of being seen as a standard preconception test, which would restrict the subject's freedom of choice. Therefore, one should determine whether the program is compelling people to take the test before implementing it (Dive and Newson 2022). This freedom only distinguishes eugenics and RCS; therefore, it must be taken care of. Ethical concerns also arise when conditional selection leads to a smaller number of diseased individuals of that type, rendering society less accepting of the affected few (Dive and Newson 2022).

18.5 Ethical Issue of Reproductive Autonomy

Every individual likes to have the freedom to make important decisions regarding whether to reproduce or not, how many children to have, when to have children, whom to reproduce with, etc. (Purdy 2006). These choices are very important to individuals; therefore, while screening for a genetic test, one should make sure that his or her freedom is protected. But deciding its boundaries is quite crucial. Some feel the extent of reproductive autonomy should be unrestricted, while others support the boundaries of reproductive autonomy. Some opine that the use of technology to decide the kind of upcoming generation is morally unacceptable and ethically unsound. While others say that it is better to have screening than to have a diseased child. Boundaries to reproductive autonomy can be determined if the choices they make will harm the other individual, and if that is the case, the nature and magnitude of harm should be justified (Chokoshvili et al. 2018).

The above level or sense of harm can be explained by taking the example of the Veneto study, which was analyzed for its different ethical perspective (Bruni et al. 2012). Bruni et al. (2012) mentioned some of the ethical issues that occur due to policies and individual choices during carrier screening of CF with the help of a previous study (Castellani et al. 2009), which was on the population-level effect of choices of CF screening in Vento, Italy, during 1993–2007 (Bruni et al. 2012). This study stated that children born will not complain about their parents' reproductive choices, as if they had taken care of it, that child would not have existed at all. In other words, CF-affected children are not harmed by their parents' reproductive choices. It is also important to consider if individual decisions will harm other individuals, such as increasing healthcare expenditure due to a greater number of affected children. But these considerations do not justify the conclusion that carrier

couples who are ready to bring an affected child are doing something wrong. According to one of the views, it is completely fine to pose a high risk or cost to others because of one's reproductive decision. At the same time, it cannot be neglected that the decisions taken by each couple determine how society will live in the future. When an individual imposes risk because of a morally wrong decision, he can justify the risk and cost by citing various reasons that forced him to take such a decision (Chokoshvili et al. 2018; Bruni et al. 2012; Buchanan et al. 2001). Individual views about abortion, embryo selection, heterologous fertilization, and moral values determine the degree to which ethical issues can arise. Those who see risk and cost to others due to carrier couples' reproductive decisions are indirectly saying that natural conception is morally wrong for such couples. Some people cannot afford assisted reproduction, preconception screening, or prenatal screening, and a strong desire to have a child cannot be ignored in the absence of these factors. Coercing carrier couples not to reproduce naturally or forcing them to adopt or undergo ART is challenging their reproductive autonomy. It has also been suggested that, despite infringing, one should focus on ways to prevent or reduce the social harm their decisions may cause (Bruni et al. 2012).

18.6 Ethical Issue on Disabled

In the entire chapter, we have discussed the ethical issue of screening carrier couples and its impact on society. What about people who are already suffering from various genetic diseases? Elimination of affected fetuses or avoiding conception by carrier couples may result in a diminished number of affected individuals. This practice, if followed strictly, may result in diminishing the number of diseased individuals, which may leave the public less sensitive to helping affected individuals (Bruni et al. 2012; Parens and Asch 2000). In short, a disease may become stigmatized or orphaned, and the affected individuals may be looked down upon with a negative judgment. Therefore, to avoid such ethical issues, a few programs should also focus on working for the individuals that are already affected to remove the stigma they face.

18.7 Conclusions and Future Perspective

In this chapter, we have tried to highlight all the possible points at which reproductive carrier screening can have ethical issues. Right from screening all the way up to reporting and counseling, there is a high possibility of having ethical concerns. Uncertainty of a variant and choosing the panel of diseases to be screened might unnecessarily trouble a population that actually might not have much relation to the disease and thus interfere with the ethics. The pathogenic nature and penetrance of all variants are not well known and can vary significantly from one population to the other depending on the genetic background. Genes that show pathogenic mutations also harbor variants of unknown significance in other individuals, making it very

difficult to assign pathogenicity to such variants. During genetic counseling upon screening, the counselor's advice can give rise to an ethical issue. Directional counseling may influence the reproductive decisions of patients toward embryo selection or abortion. Sometimes, the counselor faces ethical issues while helping patients make reproductive decisions that interfere with the norms of society and religious beliefs. Another important ethical issue in reproductive carrier screening is its objective, which overlaps with eugenics by manipulating heredity and interfering with the genetic pool of humans. In some cases, the subjects in carrier screening tend to feel forced to make reproductive decisions, thus hampering their reproductive autonomy. The ethical issue is a very important part of the challenges in carrier screening, as any action taken by the subjects can result in legal issues. Therefore, for easier adoption, a screening program should be designed keeping in mind various ethical issues that exist and avoiding adding new ones to the list.

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Genetic Counseling in Reproductive Medicine

19

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Abstract

Genetics is a rapidly changing landscape, and its applications have expanded into many areas of medicine, including human reproduction and infertility. This chapter imparts knowledge in the field and specialty of genetic counseling in reproductive medicine. Infertility genetic counselors work with patients struggling to become pregnant who desire preconception, prenatal, or preimplantation genetic testing or carrier screening. Common genetic disorders of male and female infertility, recurrent pregnancy loss, and available and emerging diagnostic tools are reviewed, and the importance of genetic counseling in each scenario is discussed. Genetic counseling helps patients/couples or families understand the basics of their condition and provides information related to the condition, such as available reproductive options, which helps them make informed decisions and empowers them to advocate for themselves.

Keywords

Genetic counseling · Infertility · Single gene disorder · Recurrent pregnancy loss · Genetic diagnosis · Reproductive medicine · Genetic testing

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19.1 Introduction

Reproductive genetics, in addition to prenatal screening and diagnostic regimes, encompasses preconception genetic counseling, assisted reproductive technologies (ART), and preimplantation genetic testing (PGT). Identifying individuals at risk of having children affected by genetic disorders or congenital anomalies allows counseling that aims at informed decision-making. This process occurs at the preconceptional or prenatal stage. However, there are more options when risk is identified before pregnancy.

The Genetic Counseling Definition Task Force of the National Society of Genetic Counselors (NSGC) developed the following definition of genetic counseling, which was approved by the NSGC Board of Directors in 2006: Genetic counseling is the process of helping people understand and adapt to the medical, psychological, and familial implications of the genetic contributions to diseases. This process integrates the following: Interpretation of family and medical histories to assess the chance of disease occurrence or recurrence; education about inheritance, testing, management, prevention, resources, and research. Counseling promotes informed choices and adaptation to the risk or condition (Resta et al. 2006).

Genetic counselors have advanced training in medical genetics and can guide and support patients seeking more information about how inherited conditions might affect them or their families and how to interpret genetic test results based on personal and family history.

19.2 Prenatal Genetic Counseling

Pregnancy can be an exciting time for parents but also a time of uncertainty, especially if there are concerns about the genetic health of the pregnancy. Prenatal genetic counseling is a professional assessment of risk factors that the couple's family might have, as well as their medical and pregnancy histories. It can reveal the underlying genetic causes of any anomalies in the pregnancy and define the risks for diseases that could be passed on to the child.

19.3 Preconception Genetic Counseling

Planning a family is a time of great delight and anticipation. Expectant parents want the safest pregnancy possible for both the mother and the child. One of the best times to test for common genetic disorders and understand the inheritance risk of a condition that runs in the family is before conception. Whether couples are facing a known genetic condition or have no known risks, preconception genetic counseling can be both informative and reassuring.

19.4 When to Offer Genetic Counseling to a Couple

- Is concerned about a sickness or hereditary issue that runs in their family.
- Has a genetically compromised child and intends to get pregnant in the future.
- Has a family history of children born with a birth defect or intellectual disability.
- Is having trouble conceiving.
- Has suffered one or more pregnancy losses (miscarriages, stillbirths, or ART cycle failures).
- Has obtained abnormal prenatal screening or ultrasound results.
- Is worried about the hazards of advanced parental age.
- The baby is expected to be born with a birth defect or genetic disorder.

19.5 Genetic Counseling in Male Infertility

Male factors contribute to about 50% of the cases of infertility, and approximately 7% of the male population experiences infertility.

19.5.1 Klinefelter Syndrome (47, XXY) and Jacob Syndrome (47, XYY)

Klinefelter syndrome (47, XXY) and Jacob syndrome (47, XYY) are common sex chromosomal abnormalities in males, each occurring in approximately 1–2 in 1000 live births. Most males with Klinefelter syndrome are only identified when they fail to conceive and undergo fertility assessments. About 11% of individuals with azoospermia have a nonmosaic form of Klinefelter syndrome, and mosaic individuals often present with oligozoospermia (Van Assche et al. 1996). However, Klinefelter syndrome has phenotypic variability.

Individuals with Jacob syndrome (47, XYY) are mostly fertile but possess an increased risk of infertility compared to karyotypically normal 46, XY males. Successful recovery of spermatozoa in some patients is used in ICSI procedures to achieve a successful pregnancy. These couples should undergo genetic counseling to obtain the right information available, including their risk of embryonic and fetal aneuploidy, alternatives for preimplantation and prenatal diagnosis, and the necessary support and reassurance (Yahaya et al.2021).

19.5.2 Y-Chromosome Microdeletions

One of the most commonly identified genetic causes of male infertility is Y-chromosome microdeletions (YCMD). Azoospermia factors (AZFa, AZFb, and AZFc), three spermatogenetic loci, have been located on the long arm of the Y chromosome. Deletions in these regions can result in varying degrees of spermatogenic failure, contributing to infertility (Fig. 19.1). About 4% of males with

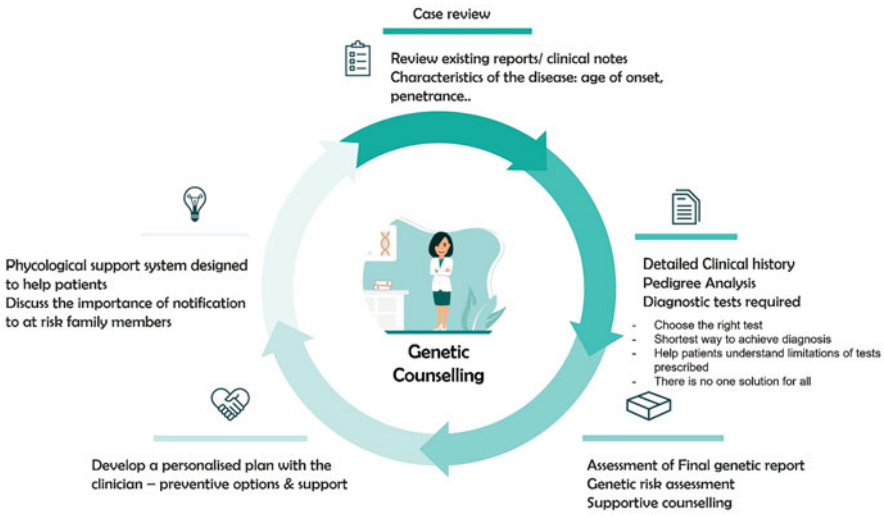

Case review
Review existing reports/ clinical notes
Characteristics of the disease: age of onset, penetrance..
Phycological support system designed to help patients
Discuss the importance of notification to at risk family members
Genetic Counselling
Detailed Clinical history
Pedigree Analysis
Diagnostic tests required
- Choose the right test
- Shortest way to achieve diagnosis
- Help patients understand limitations of tests prescribed
- There is no one solution for all
Develop a personalised plan with the clinician – preventive options & support
Assessment of Final genetic report
Genetic risk assessment
Supportive counselling

Fig. 19.1 The process of genetic counseling

Table 19.1 Semen phenotype and TESE outcomes in different types of AZF microdeletions. *AZF* azoospermia factor region, *TESE* testicular sperm extraction (Cioppi et al. 2021)

Regions	Deletion size	Semen phenotype	TESE outcome
AZFa	AZFa 0.8 Mb	Azoospermia	Virtually no chance
AZFb	AZFb 6.2 Mb	Azoospermia/Cryptozoospermia	Virtually no chance (sY 1192-) Positive (sY 1192+)
AZFc	AZFbc 7.0–7.7 Mb	Azoospermia	Virtually no chance
AZFc	AZF c 3.5 Mb	Azoospermia/Oligozoospermia	Positive (50–60%)

oligozoospermia, 14% of those with severe oligozoospermia, and 18% of those with nonobstructive azoospermia have YCMDs (Simoni et al. 2004). When sperm is available for ICSI in individuals with Yq microdeletions, it is important to provide genetic counseling for the couple, as the microdeletion will be passed on to all of their male offspring, and they may face similar fertility issues. Testing for YCMD can also aid in planning interventions and predicting the possibility of successful sperm retrieval by testicular sperm extraction procedures. The possibility of retrieving sperm following positive YCMD results is described in Table 19.1.

19.5.3 Cystic Fibrosis

In total, 1–2% of males with infertility and 6% of obstructive azoospermia cases are found to have congenital bilateral absence of the vas deferens (CBAVD) (Babul-Hirji et al. 2021). CBAVD is observed in almost 95% of men with cystic fibrosis

(CF), an autosomal recessive disorder. Cystic fibrosis Transmembrane Conductance Regulator (CFTR) gene mutations can cause CBAVD, which is a monosymptomatic form of CF. Typical cystic fibrosis is characterized by pancreatic insufficiency, high electrolyte concentrations in sweat (salty sweat), chronic pulmonary obstruction, male infertility, and other abnormalities. When CBAVD is the only phenotype found in a patient with at least one mutation in the *CFTR* gene, it is known as the genital form of cystic fibrosis (CF) or CF-CBAVD. With the availability of assisted reproductive techniques (ART), like testicular or epididymal sperm aspiration, intracytoplasmic sperm injection, and in-vitro fertilization, it is possible that men with CBAVD can have children. Genetic counseling should be offered to these couples undergoing ART to counsel them on the probability of having offspring that carry *CFTR* gene mutations. Genetic testing must be done on one of the partners, ideally the infertile one, and if a mutation in the CFTR gene is discovered, the other partner should also be tested to determine the likelihood that their children will have cystic fibrosis (De Souza et al. 2018).

19.6 Genetic Counseling in Female Infertility

About 10% of women of reproductive age are unable to get pregnant or have a live birth. Female factors contribute to at least 35% of infertility cases and include a wide range of causes that affect ovarian development, maturation of oocytes, fertilization, implantation, and growth.

19.6.1 Triple X Syndrome (47, XXX)

Triple X syndrome (47, XXX), also called trisomy X syndrome, is a sex chromosome aneuploidy in which a female has one extra X chromosome. It is the most common female chromosomal abnormality, affecting about 1 in every 1000 female newborns. Trisomy X syndrome is usually not inherited, mainly because of maternal nondisjunction during meiosis. Mosaic 46, XX/47, XXX is found in 20% of cases. Women with Triple X syndrome are often fertile and produce babies with a normal chromosomal number. Some cases of primary infertility with premature ovarian insufficiency (POI), amenorrhea, and premature menopause are observed. Patients with trisomy X can present with various characteristics, and careful genetic counseling is needed for pregnant women and their partners following a prenatal diagnosis of trisomy X.

19.6.2 Turner Syndrome (45/X)

Turner syndrome, also known as monosomy X, is a female-only genetic disorder. In total, 1/10th of spontaneously aborted fetuses are found to have Turner syndrome. It occurs when one X chromosome is completely or partially deleted in females; thus,

the karyotype is 45, X. Other variants of Turner Syndrome include mosaic karyotypes (for example, 45, X/46, XX and 45, X/47, XXX), an isochromosome of either the p or q arm, ring chromosomes, and the presence of Y chromosome along with X. Approximately 40–50% of women with TS have a 45, X karyotype, 15–25% have mosaicism (45, X/46, XX), 20% have an isochromosome, and ring X chromosomes are less common. Most Turner Syndrome patients exhibit delayed puberty, ovarian dysgenesis, hypogonadotropic hypogonadism, ambiguous infantile external genitalia, and infertility. Typical physical features associated with TS include short stature, a webbed neck, low-set ears, and swollen hands. Ovarian failure is a typical feature of Turner syndrome (TS), and the patients are followed clinically with hormone replacement therapy (HRT). For patients with spontaneous puberty, genetic counseling regarding preimplantation, prenatal diagnosis, and the donor-oocyte option is indicated. Patients with dysgenetic gonads and a Y chromosome have an increased risk of developing gonadoblastoma. It is important to perform a gonadectomy before HRT and pregnancy with oocyte donation.

19.6.3 Fragile X Syndrome

Fragile X syndrome is the most common inherited cause of intellectual disability and the most common genetic cause of autism. The disorder is caused by the expansion of cytosine–guanine–guanine (CGG) trinucleotide repeats or mutations in the fragile X messenger ribonucleoprotein-1 (FMR-1) gene. The FMR-1 gene typically has 40 or fewer CGG repeats and is found on the X chromosome at Xq27.3 (Fig. 19.2). The phenotypic consequences of Fragile X are categorized as normal, premutation, or full mutation based on the quantity of nucleotide repeats. Autism and intellectual disability are caused by the fully expanded form of the mutation, whereas macroorchidism after puberty and premature ovarian failure (POF) are caused by the premutation known as fragile X-associated tremor/ataxia. Women with an

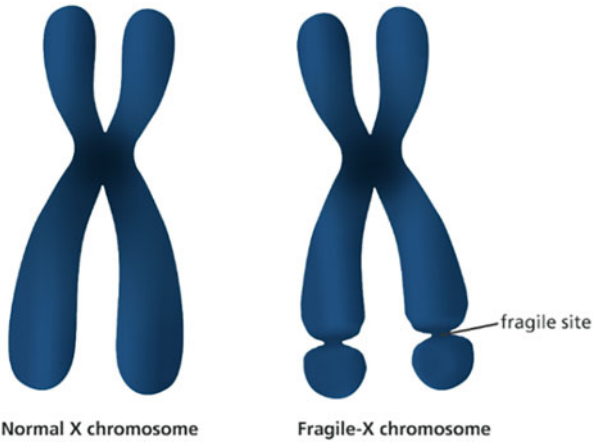


Fig. 19.2 An illustration showing the appearance of normal and Fragile-X chromosomes. Image credit: Genome Research Limited

FMR-1 premutation frequently experience premature ovarian failure. Premutation terminology refers to the expansion of repeats in the FMR-1 region CGG (61–200) repeats. Infertility may be impacted by Fragile X, which is a key contributor to POF and irregular menstruation. Normal phenotypes are normally displayed by premutation carriers, but they pose the danger of developing into complete mutations in the next generation. The prevalence of women with POF who have the premutation is estimated to be between 13 and 26%. Among women with sporadic POF, 0.8–7.5% have subsequently been found to be premutation carriers, and up to 13% of women with familial POF carry the premutation (Martin and Arici 2008). Individuals with CGG repeats above 200 are considered a full mutation. These individuals cannot make the FMR-1 protein and have fragile X syndrome. As the fragile X mutation is located on the X chromosome, it follows the classic pattern of X-linked inheritance. Women who carry the mutation will transmit it to 50% of their offspring. Men who carry the mutation will transmit it to all of their daughters but none of their sons. The risk of the expansion of a premutation into a full mutation depends on the size of the repeat that is carried by the mother.

In total, 5–10% of these carriers are able to conceive without medical intervention. Couples who carry the premutation or full mutation should be offered genetic counseling prior to conception. It is important to identify and recognize clinical situations that warrant fragile X testing. Genetic counselors can counsel and inform patients about their reproductive possibilities and ways to increase their chances of a successful reproductive outcome.

19.7 Single Gene Disorders

Genetic syndromes that affect male or female fertility include Kartagener's syndrome, myotonic dystrophy, Noonan syndrome, Fanconi anemia, sickle cell anemia, beta-thalassemia, etc. Other notable conditions include disorders of sex development (mutations in the *DAX1, CBX2, SRY, SOX9, and RSPO1* genes), reproductive dysgenesis disorders (mutations in the *AMH, AMHR2, ARX, DHH, NR5A1, WNT4, WT1* genes), hypogonadotropic hypogonadism and Kallmann syndrome (mutations in the *KAL1, FGFR1, PROKR2, GNRH1, TAC3, LEP, NSMF, CHD7, DAX1, KISS1* genes), ambiguous genitalia, and androgen insensitivity (mutations in the *AR* gene). Endocrine defects are caused by *CYP17, CYP21, and CYP21A2* mutations. Also, various metabolic defects (e.g., galactosemia) and mutations in the mitochondrial energy pathways (*POLG1* and mitochondrial DNA genes) cause toxicity, affecting female or male fertility (Hyde and Schust 2015; Yatsenko and Rajkovic 2019). *AURKC* (aurora kinase C) is highly expressed in the testis. Mutations in the gene were reported in several infertile males with abnormal sperm morphology and sperm polyploidy. *FOXL2* encodes an ovarian development–specific transcription factor. Studies have found that dominant mutations in *FOXL2* cause premature ovarian failure (Harris et al. 2002).

Comprehensive genetic screening by next-generation sequencing helps identify couples who may be asymptomatic carriers for these conditions and helps plan

interventions accordingly. This can also aid in risk assessment and identification of couples who can be offered preimplantation genetic testing for monogenic disorders (PGT-M) or donor gamete options based on the inheritance pattern of the identified mutation or plan prenatal interventions. Identification of pathogenic mutations in any of these genes can be used to offer genetic counseling to these patients regarding the possibility of their transmission to the next generation.

19.7.1 Kartagener's Syndrome

An autosomal recessive genetic disease called Kartagener's syndrome (KS) accounts for about 50% of cases of primary ciliary dyskinesia. (PCD). Situs inversus, sinusitis, bronchiectasis, and infertility are all its symptoms. Two of the most thoroughly researched genes in PCD/KS are the axonemal dynein intermediate chain gene DNAI1 (located on chromosome 9p12–21) and the axonemal dynein heavy chain gene DNAH5 (located on chromosome 5p15–14). Due to the dyskinetic ciliary activity in the fallopian tubes, women with Kartagener's syndrome (KS) are at risk for infertility. For patients with tubal factor infertility, in vitro fertilization–embryo transfer (IVF-ET) is seen as the most appropriate type of treatment (Abu-Musa et al. 2008).

The sperm of infertile male patients with PCD/KS is usually immotile to varying degrees or is even completely static and manifests defective morphology. Afflicted men generally have immotile ejaculated sperm and have almost no chance of achieving a spontaneous pregnancy. For these PCD/KS patients, assisted reproductive technology, including in vitro fertilization and ICSI, should be considered. In vitro fertilization could be a treatment option for patients when sperm motility is retained. However, ICSI is currently the only treatment option for most PCD/KS patients. ICSI overcomes the factors related to impaired motility and bypasses the natural processes for fertilization. A number of reports have confirmed that it is possible to obtain healthy offspring in PCD/KS patients with the help of the ICSI technique (Sha et al. 2014).

19.7.2 Myotonic Dystrophy 1

Myotonic dystrophy 1 (DM1) is defined as a hereditary, autosomal dominant multisystem disorder characterized by the development of structural and functional abnormalities in the muscle membrane protein associated with muscular dystrophy, cardiac conduction disorders, cataracts, mental retardation, and endocrine and reproductive defects. The genetic mutation in DM1 is the expansion of a CTG repeat sequence found in the 3′-untranslated region of the myotonic dystrophy protein kinase (DMPK) gene located on chromosome 19q13.3. A common finding in DM1 is abnormalities in the reproductive system. A significant trait that affects about 80% of people is progressive testicular atrophy. Hyalinization, atrophy, fibrosis of the seminiferous tubules, and a decline in the amount of sperm are examples of

histological abnormalities. Oligospermia and azoospermia are reported in approximately 73% of DM1 patients (Kim et al. 2012).

19.8 Genetic Counseling in Recurrent Pregnancy Loss

The American Society for Reproductive Medicine (ASRM) has recently redefined recurrent pregnancy loss as two or more pregnancy losses. A pregnancy loss is defined as a clinically recognized pregnancy involuntarily ending before 20 weeks. A clinically recognized pregnancy is when the pregnancy has been visualized on an ultrasound or when pregnancy tissue has been identified after a pregnancy loss.

19.8.1 Chromosomal Abnormalities

Couples with cytogenetic abnormalities account for approximately 2–5% of RPL cases, with balanced Robertsonian and reciprocal chromosomal translocations being the most common chromosomal abnormalities. A reciprocal translocation is the exchange of chromosomal material between two nonhomologous chromosomes. If the long arms of acrocentric chromosomes join, the rearrangement is called Robertsonian (ROB) translocation. The formation of balanced, unbalanced, and normal gametes is dependent on the breakpoints and the chromosomes involved. The lesser imbalance raises the possibility of having children with unbalanced translocations, while the bigger imbalance will result in miscarriages. Balanced translocations may also cause sequence rearrangements of functional genes, which may result in reproductive errors causing repeated pregnancy losses.

Chromosomal inversion is a common structural rearrangement of chromosomes where the chromosome undergoes two breakages involving three parts. The central fragment rotates 180° before returning to its original position without losing any genetic material detectable through karyotyping. Most inversion carriers show no phenotypic abnormalities but are likely to produce unbalanced gametes that might cause infertility, miscarriage, stillbirth, and an abnormal karyotype in the offspring. Pericentric inversion of chromosome 9 (inv[9][p11q13]) is the most common inversion due to its structural organization, making it more prone to breakage. The risk of pericentric inversions producing unbalanced progeny is about 10% for female carriers and 5% for male carriers. Due to meiotic changes, inversion carriers are at high risk of gamete production failure and/or unbalanced gametes that can lead to subfertility. The chromosomes of embryos fertilized by abnormal gametes can result in partial monosomy or trisomy. Genetic counseling should be offered to inversion carriers with a history of adverse pregnancy outcomes, such as miscarriage with chromosomally abnormal embryos. Preimplantation genetic testing for structural rearrangement (PGT-SR) can be considered to prevent repeated abortions (Shao et al. 2020).

Polymorphism variations mainly refer to variants in the chromosomal heterochromatin region. Increased lengths of the heterochromatic regions on the long arms of

these chromosomes are designated as 1qh+, 9qh+, 16qh +, and Yqh+. Sometimes, the heterochromatin is reduced in these chromosomes, referred to as 1qh−, 9qh −, and 16qh−. Increased lengths of the short-arm satellites and stalks of the acrocentric D and G group chromosomes (13, 14, 15, 21 and 22) are designated as 14 ps + and 13pstk+, while increased lengths of the short arms themselves are designated as p + (e.g.15p+). Despite the fact that polymorphisms in heterochromatic regions are regarded as normal variations in humans, a high frequency of chromosomal variants in infertile patients may support the theory that large heterochromatic blocks may disrupt chromosome pairing and result in meiotic arrest, which would lead to infertility. Heterochromatin plays an essential role in spindle attachment and chromosome movement, meiotic pairing, and sister chromatid cohesion. When chromatin variation occurs in these regions, it causes defects in centromere function and kinetochore assembly, difficulty in homologous chromosome pairing, and impacts cell division, thus affecting gamete formation. Polymorphic variants in the satellite region or the acrocentric chromosomes may favor recombination between nonhomologous acrocentric chromosomes, leading to Robertsonian translocation.

19.8.2 Genetic Thrombophilia and RPL

The role of genetic thrombophilia in women with recurrent pregnancy loss has been studied, and it is likely a causative factor for recurrent pregnancy loss and other reproductive complications. Thrombophilia is a group of genetic disorders that cause abnormal blood clots. Thrombophilia is associated with recurrent pregnancy loss, fetal growth restriction, late miscarriages, stillbirth, and preeclampsia. A successful pregnancy needs efficient uteroplacental circulation. It was hypothesized that maternal thrombophilia may be a risk factor for preeclampsia and intrauterine growth retardation since this may be altered by disorders linked to a prothrombotic condition. The most common mutations associated with thrombophilia and RPL are the Factor V Leiden mutation, prothrombin mutation, protein C, protein S, antithrombin deficiency, and *MTHFR* mutation (Voicu et al. 2020).

19.9 Genetic Testing in RPL

Genetic testing for recurrent pregnancy loss is usually advised after the first or second pregnancy loss. Identification of the etiology of pregnancy loss forms a critical basis for how interventions are planned to ensure the safety and health of future pregnancies. It also helps in the evaluation of recurrence risk.

19.9.1 Product of Conception

Sporadic fetal aneuploidy is the most common cause of spontaneous pregnancy loss, particularly in the first trimester of pregnancy. Some couples with recurrent

pregnancy loss have additional genetic factors, and others have nongenetic etiologies. Genetic testing of the products of conception in couples experiencing two or more pregnancy losses may assist in identifying the underlying causes and in counseling patients about their prognosis in a subsequent pregnancy. Genetic counseling is essential in deciding the most appropriate test to be applied to a product of conception. A detailed evaluation of family history, consanguinity, and previous pregnancy loss plays a vital role in deciding the test to be applied. In addition, genetic counseling also helps the couple come to terms with the trauma of pregnancy loss and can encourage good mental health while moving forward.

The most comprehensive test presently applied to testing POC samples is a chromosomal microarray. The advantage of this test is that it can evaluate the molecular karyotype and simultaneously identify copy number variations (CNVs) caused by insertions and deletions across the genome. Phenotypic attributes play a critical role in the interpretation of insertions and duplications found in microarray results. Follow-up genetic counseling is always advisable, even in cases where microarray results are found to be normal. This is because microarrays cannot detect balanced translocations and mutations.

19.9.2 Parental Karyotyping

Karyotyping in couples with recurrent pregnancy loss is recommended to identify if they are carriers for any structural rearrangements like balanced translocations (Fig. 19.3) and inversions, which increase the likelihood of recurrent fetal aneuploidy and may direct interventions. This should be followed by genetic counseling. Genetic counselors can counsel the affected couple about their chances of transmitting the identified abnormality, their chances of future pregnancy loss, and other reproductive options like preimplantation genetic testing for structural rearrangements (PGT-SR).

Robertsonian translocations result from the breakage of two acrocentric chromosomes at or close to their centromeres, with a subsequent fusion of their long arms to form one chromosome. The total chromosome number in a Robertsonian translocation carrier is therefore reduced to 45. Chromosomes involved in Robertsonian translocations are 13, 14, 15, 21, and 22. Robertsonian translocations occur with high frequency in the general population, with an incidence of approximately 1 in 1000, the commonest being fusion between chromosomes 13 and 14. Robertsonian translocation carriers are asymptomatic but often produce unbalanced gametes, which can result in monosomic or trisomic zygotes. These patients can benefit from PGT-SR. Genetic counseling in such cases helps patients understand the implications of PGT-SR and prepare for the possibility of aneuploidy in embryos, as Robertsonian translocations are associated with a high incidence of aneuploidy.

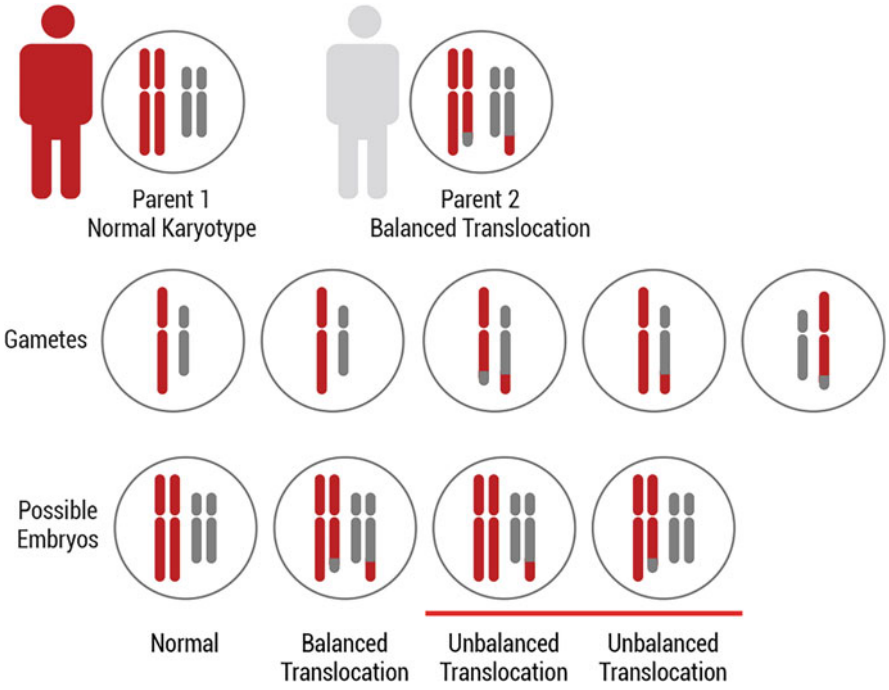


Fig. 19.3 Inheritance of balanced translocation. Image credit: Progenesis

19.9.3 Carrier Screening

With the advancement and availability of technologies like next-generation sequencing, it is now possible to screen the coding region or entire genome for genetic mutations. A number of genetic disorders occur more frequently in certain ethnic populations or couples with a family history of a genetic condition. Therefore, before conception, a couple can undergo carrier screening to identify if both are carriers for mutations for an autosomal recessive disorder or X-linked disorders.

If an asymptomatic couple is a heterozygous carrier for the same mutation, in each pregnancy there is a 25% risk of being affected by an autosomal recessive disorder. In cases where one of the parents is a carrier and the other is a noncarrier for an autosomal recessive mutation, the probability of having carriers and a normal genotype is 50%. Hence, in such cases, interventions are not necessary. If any one parent is a carrier of an autosomal dominant mutation, there is a 50% possibility of the recurrence of the condition in the next generation.

As per the ACMG guidelines, prenatal and preimplantation interventions are warranted only in cases where pathogenic and likely pathogenic variants have been identified. Variants of unknown significance lack information on the pathogenicity of the mutation. In cases where VUS is identified, it is important to perform segregation analysis in affected and unaffected individuals in the family to determine

Table 19.2 Pretest and posttest genetic counseling session

Pretest genetic counseling	Posttest genetic counseling
Evaluate the family history and medical records	Interprets the test results
Helps the individual or family understand about the genetic condition	Discusses the need for further testing
Explores the option available for genetic testing, reliability and limitations	Explains the inheritance pattern and the risk of recurrence depending on the results and final diagnosis
Discusses the risk and benefits of genetic testing	Discusses the available management and preventive options
Considers the inheritance pattern and the risk of recurrence	Addresses the emotional and social concerns
Declares the possible uncertainties due to present lack of knowledge	Discusses the implications of the reports to the individual and the family
Discusses reproductive options	Refers to other resources or support groups for help

if the VUS is benign or pathogenic. Based on this information and phenotype–genotype correlation, the variants are reclassified, which then guides interventions.

Pre- and posttest genetic counseling should be offered to couples undergoing carrier screening. The couple should be made aware of the benefits and limitations before testing, the risk of inheritance, the clinical significance of the findings along with best reproductive decisions after testing to make informed decisions (Table 19.2).

19.9.4 Congenital Anomalies

Congenital anomalies are often caused by chromosomal, monogenic, and multifactorial disorders. Aneuploidies are the most frequent chromosomal anomalies. Due to their high incidence and capability of viable birth with multiple congenital anomalies, three complete trisomies hold significance for the prenatal diagnosis: trisomy 13, trisomy 18, and trisomy 21. Genetic counseling is crucial in such cases, where the couple should be informed of the prognosis to make informed decisions. Genetic congenital anomalies are an important part of the clinical presentation of monogenic diseases. In total, 7.5% of isolated or syndromic congenital anomalies are caused by monogenic disorders (Wieacker and Steinhard 2010). Congenital anomalies can be detected prenatally or at birth, and sometimes, they are apparent only in later development. The likelihood of recurrence will be estimated according to the inheritance pattern of the disease with a known diagnosis. A phenotype must be associated with a specific disease when a definite diagnosis is not available. The risk of expressing a monogenic disease is dependent not only on the pattern of inheritance but also on other factors such as the incidence of the disease, the presence of other affected members, the penetrance and variable expressivity, ethnicity, and the influence of environmental factors.

19.10 Genetic Counseling for Preimplantation Genetic Testing

It is essential to receive genetic counseling prior to preimplantation genetic testing in order to clarify the test's goal and go through its advantages, risks, and restrictions. It is to ensure that patients have the necessary information to make informed decisions that are consistent with their reproductive plans and values. This section discusses considerations and genetic counseling points associated with PGT-A, PGT-SR, and PGT-M.

19.10.1 Preimplantation Genetic Testing for Aneuploidy (PGT-A)

PGT-A results are classified into euploid (normal number of chromosomes), aneuploid (aberrant number of chromosomes), and mosaic (combination of normal and abnormal chromosomes). Aneuploidies like autosomal monosomies are lethal and are unable to produce viable pregnancies. Other aneuploidies, such as trisomies 13, 18, and 21, or those involving the sex chromosomes, can result in live births with variable and adverse phenotypes. Aneuploid embryos are mostly not recommended for transfer. Embryos with mosaic PGT-A results have been associated with reduced implantation potential and an increased risk of miscarriage. However, several live births have resulted after the transfer of these embryos. During genetic counseling, it is important to address the variability that exists within the mosaic category in order to ensure that clients have a clear understanding of the information provided by PGT-A. Genetic counseling for PGT-A must emphasize the limitations of the test. Congenital abnormalities caused by insertions or deletions below 10 Mb cannot be detected by PGT-A and are, therefore, beyond the scope of the test. Care must be taken to explain to the patient that routine pregnancy monitoring after conception with PGT is a must to ensure optimal monitoring of fetal development.

19.10.2 Preimplantation Genetic Testing for Structural Rearrangements (PGT-SR)

PGT-SR is mainly recommended for couples with chromosomal rearrangements such as a balanced translocation, a Robertsonian translocation, or an inversion. Most CNV-based NGS tests for PGT-SR cannot detect balanced translocations. Though balanced translocations are known to be benign, during genetic counseling, patients must be made aware of the fact that PGT-SR technology cannot differentiate between the euploid and balanced forms of the chromosome rearrangements (i.e., without extra or missing segments). This is because PGT-SR (like PGT-A) is typically able to detect differences only in the quantity of the DNA present. Extrarisk elements that are thought to be connected to the specific rearrangement are the dangers of balanced X-autosome translocations. After conception, a prenatal diagnosis should be provided to confirm the chromosomal complement (euploid or balanced).

19.10.3 Preimplantation Genetic Testing for Monogenic Disorders (PGT-M)

PGT-M is employed for one or more genetic variants associated with a monogenic (Mendelian) disorder. PGT-M can be pursued when an individual affected by a genetic disorder, a carrier couple (such as with an autosomal recessive condition), a male with a Y-linked disorder, or a female carrier of an X-linked condition wishes to have a healthy baby free of the hereditary condition. The outcome of PGT-M-tested embryos is reported as affected, unaffected, or carrier. During genetic counseling, the couple should be made aware of any known risks associated with carrier status. Depending on the condition and mutation, analysis often requires several weeks or months of preparation before testing embryo samples and frequently requires genetic testing of a patient's or couple's relatives, which may be difficult because of logistics or family dynamics. Although PGT-M is available for most identified genetic variants, there are exceptions. Cases in which family members are unavailable for linkage analysis may not be possible to subject to PGT-M, and certain genes may present technical difficulties. Therefore, prior consultation with a geneticist is advisable.

Since PGT-M in embryos is performed on whole genome amplified products, it is important to counsel the patient on the limitations of such testing and the risk of allele dropout (ADO). In order to overcome the limitations of allele dropout, the inclusion of informative STR markers from family members becomes necessary.

19.11 Emerging New Technologies

Prior to the rapid advancement of genomic testing, genetic testing was rarely able to deliver results quickly. However, this is now changing, and genomic data are now more frequently used to inform patient care decisions. Couples at increased risk of having offspring with a specific genetic disorder have several reproductive options aimed at avoiding the birth of an affected child.

19.11.1 Noninvasive Pre-Natal Screening (NIPS)

During gestation, the mother's blood contains a mix of cell-free DNA (cfDNA) that comes from her cells and cells from the placenta. The DNA in placental cells is identical to the DNA of the fetus. Analyzing cfDNA from the placenta helps in the early detection of certain genetic abnormalities instead of the traditional invasive procedure like chorionic villi sampling or amniocentesis. NIPS can be offered from 10 weeks of gestation age. Noninvasive prenatal testing (NIPT) has the highest sensitivity for Down syndrome (trisomy 21), followed by trisomy 18 (caused by an extra chromosome 18), trisomy 13 (caused by an extra chromosome 13), and sex chromosome abnormalities. The accuracy of the test varies by disorder. Due to technological advancement, NIPS can now detect chromosomal microdeletions,

microduplications, and monogenic disorders. NIPS is a screening test, which means it can only estimate whether the risk of having certain conditions is increased or decreased. An invasive prenatal diagnosis by chorionic villi sampling or amniocentesis should be performed to confirm the diagnosis.

Genetic counseling enables the pregnant woman and her partner to make an informed choice and provide consent for either NIPT or invasive prenatal diagnosis (PND) or refrain from further testing. The eventual decision should reflect the principles of informed choice and be based on relevant knowledge, consistent with the couple's values, and behaviorally implemented, i.e., the decision must be documented, and when requested, a test must be performed. During genetic counseling, information is provided about the benefits and limitations of the NIPT and invasive PND, as well as the potential to detect findings other than the indication for testing (van der Steen et al. 2019).

19.11.2 Whole Exome/Genome Sequencing

Whole exome sequencing (WES) comes forth as an efficient tool for molecular investigations in patients with syndromes of unknown etiology. Whole-exome sequencing (WES) is used for the clinical diagnosis of rare Mendelian disorders, especially when standard tests have negative results. WES is reported to have a molecular diagnosis rate of approximately 25%. Whole-genome sequencing (WGS) is becoming an attractive alternative due to its broader coverage and decreasing cost. WGS can detect potential disease-causing mutations outside the WES regions, particularly those due to single nucleotide variations (Volk et al. 2015).

Genetic counseling for genome-wide sequencing should involve a detailed family history and explanation of the method of testing used, its risks and benefits, and its potential to deliver uncertain, undefined, and difficult-to-interpret results; the possibility of secondary findings; implications for other family members, including the potential need for testing relatives; and the potential privacy implications of making a genetic diagnosis. In addition, the process should provide emotional support to the patients and families facing a possible genetic diagnosis, guide them in making informed decisions that are consistent with their own values, help families make effective use of the testing results, and decrease the risk of adverse outcomes when results are returned that differ from the family's expectations. Patients undergoing genetic testing who receive pretest and/or posttest genetic counseling show greater knowledge, a better understanding of the results, improved decision-making, decreased distress, and greater satisfaction with the testing process than patients who do not receive counseling (O'Daniel and Lee 2012).

19.11.3 Expanded Carrier Screening

In expanded carrier screening, many disorders are screened using a single sample. Companies design their own lists of disorders and offer expanded carrier screening.

This list is called a screening panel. Some such panels test for more than 100 different disorders. Screening panels usually focus on severe disorders that have adverse effects on the individual, like beta thalassemia, Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA), fragile X syndrome, and cystic fibrosis.

19.12 Conclusions and Future Perspective

Genetic counseling is an important aspect of the assessment and treatment of infertility. It has a major contribution to make in educating patients/couples about possible etiologies, testing options, and recurrence risks, as well as providing them psychosocial support. In the genetic counseling process, the timing of the disclosure of infertility is important, as the approach taken is guided by this knowledge. Emotions and psychological reactions may vary depending on whether the diagnosis is new, as in the adult male/female, or whether the revelation occurred over time in childhood and thus was included as a part of the transition counseling to adult health. Genetic counseling helps patients/couples, or families understand the basis of his/her/their condition, provides them with information associated with the condition, including the reproductive options available, such as donor programs and preimplantation genetic testing, and provides support, and empowers them to advocate for themselves or their family members.

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Challenges in Reproductive Carrier Screening

20

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Abstract

Carrier screening is a process that aims to identify carrier individuals who may inherit disease-causing alleles to their future generations. Due to the complicated nature of such programs, several challenges need to be addressed for better implementation. In this chapter, we have discussed various challenges in reproductive carrier screening, including individual and societal awareness, appropriate genetic counseling, technological advancements, the genetic makeup of populations, and societal and ethical issues. These challenges vary from population to population and country to country depending upon genetic disease prevalence, societal makeup, technological advancement, and literacy. It is imperative to understand and overcome these challenges for better implementation and adoption of reproductive carrier screening programs.

Keywords

Reproductive carrier screening · Ethical challenges · Disease screening · Genetic screening · Genetic inheritance · Genetic testing, reproductive medicine

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20.1 Introduction

Carrier screening has helped people learn about the probability of transferring genetic diseases or disease-causing alleles to the next generation. From single gene target to multiple targets and ancestry-based studies to population-based studies, the methods of screening have advanced over time (Kraft et al. 2019). With every new approach to screening, some of the hurdles have been solved, whereas others have created new challenges. In the previous chapter, we have already discussed how expanded carrier screening had overcome the drawbacks of normal/single-disease genetic screening and is more likely to be adopted in place of usual carrier screening. Generally, reproductive carrier screening now mainly refers to expanded carrier screening (ECS) due to its coverage of multiple diseases along with pan-ethnic screening. ECS is quite beneficial for various reproductive decisions, cutting out the cost of individual genetic testing and various other advantages, but does have its consequences and challenges that must be learned (Kraft et al. 2019). Challenges in screening vary from patient to patient, country to country, depending on awareness, the healthcare system, technical advancement, social perception, ethical values, and the law (Fig. 20.1).

Adopting carrier screening is a challenge even in developed countries, such as the USA, where the program was introduced about four decades ago. Lazarin and Haque (2016) reported that over 200,000 individuals received carrier screening in the USA despite 4 million annual pregnancies in 2015 (Lazarin and Haque 2016). Despite their support for expanding carrier screening and understanding its advantages, the majority of the 337 counselors surveyed in the USA and Canada in 2012 did not conduct carrier screening at their clinics (Lazarin et al. 2016). Another survey that collected the opinion of gynecologists on expanded carrier screening in the USA concluded that 40% of the participating obstetricians believed in restricting screening to individuals at higher risk, while only 15% of them had offered to screen all of their patients (Benn et al. 2014). These low numbers of participants are a matter of concern, and one needs to find loopholes that can identify challenges associated with poor screening practices (Kraft et al. 2019). According to a study on cystic fibrosis, how screening is offered influences its uptake and the challenges it faces (Bekker et al. 1993). A few of the challenges that are faced during carrier screening are discussed in depth in this chapter.

20.2 Public Interest and Its Awareness

Awareness and knowledge count as the primary challenges, which can spread with the help of direct-consumer marketing or physicians that are mediators between the patients and the companies offering these tests. Apart from ancestry-risked individuals or families with affected children, the general population is less familiar with genetic inheritance or conditions. A review that analyzed published data for the factors associated with the acceptance and denial of cystic fibrosis screening found a lack of knowledge or awareness as one of the major factors (Chen and Goodson

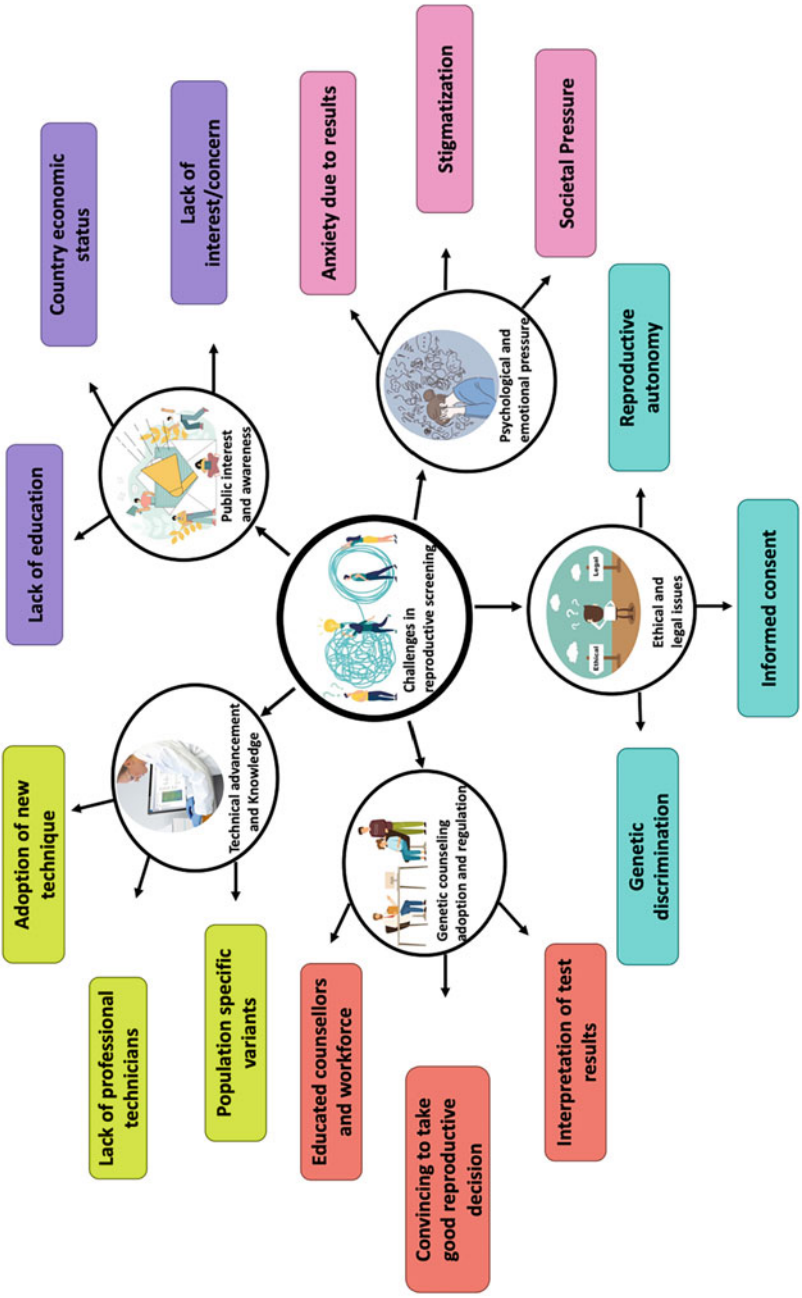


Fig. 20.1 Challenges in reproductive carrier screening

2007). Another survey of 777 Swedish couples found that 1/3 of them were interested in preconception screening, 1/3 were not, and the rest were uncertain (Ekstrand Ragnar et al. 2016). This survey also found that poor awareness about carrier screening was the reason behind the low acceptance of screening tests (Ekstrand Ragnar et al. 2016). Those who took the screening test were concerned about their future child having the disease, others who denied screening had no interest in knowing their carrier status and were not willing to go for an abortion (Plantinga et al. 2016; Holtkamp et al. 2016; Ekstrand Ragnar et al. 2016). Another survey of 240 women who were planning for pregnancy in the USA found a lack of time or interest in getting screened, while a few others did not want to cause themselves anxiety by knowing the results of carrier screening (Gilmore et al. 2017). Similarly, another survey on the views of pregnant women on expanded carrier screening in the USA found a decline in screening primarily because knowing the result wouldn't change anything except heightening their anxiety (Propst et al. 2018). Thus, all these studies concluded that the reason for denial of carrier screening is a lack of awareness, no possibility of correcting genetic changes, and heightened anxiety if the results were positive.

To tackle the above issue, emphasis on awareness can be put through primary care providers or camps targeting high-risk ethnic groups. For example, creating awareness about Tay–Sach's disease in the Jewish population, hemoglobinopathies in the Netherlands, and diseases linked to consanguineous marriages in communities practicing it (Weinreich et al. 2009). Some countries have taken the initiative to make people aware of screening programs at the premarital stage, like Cyprus, Italy, Greece, Canada, and France, especially for thalassemia (Cousens et al. 2010). People lack interest in such screening programs because of their socioeconomic status as well. Usually, surveys that were done include patients having high socioeconomic status or having high-quality medical care (Kraft et al. 2019). Creating awareness among people who lack such facilities and finding how they interpret or receive carrier screening is a challenge. People in developing countries where the majority of them do not have access to basic medical facilities might have never heard of such screening availabilities. High school awareness programs and social awareness camps that usually take place in developed countries must also be adopted by developing nations to overcome these challenges.

20.3 Psychological and Emotional Pressure Created By Screening

Routine use of screening in countries might be unwelcome by people who may feel pressurized for taking up the test. Ancestry-based screening sometimes also led to psychological pressure, according to a survey on Dutch Jewish people, which found that 26% of the participants agreed to have felt forced for being tested and 24% agreed to have undergone the test forcefully due to healthcare workers (Holtkamp et al. 2016). Screening tests for couples before marriage are a great challenge and if tested positive, they also tend to face societal and emotional pressure, thus creating

stigmatization. Reproductive-age individual carrier testing creates psychological pressure and feeling of being different from the rest. This psychological impact of screening is a negative outcome that must be kept in mind. A Melbourne high school Tay–Sach's disease carrier screening was reevaluated for its long-term impact keeping in mind the psychological effect on the subjects, and several parents admitted their concern regarding the psychological impact of genetic screening on their adolescent children (Curd et al. 2014). Different psychological responses, like questioning self-efficacy, self-concept, and anxiety, may occur due to improper carrier screening program design, its presentation, awareness, and counseling (Archibald and Wilfond 2011). Screening also creates a fear of stigmatization due to which either screening is not taken up or results in a different self-image. Most of the time, stigmatization is a postscreening consequence (Rose et al. 2016). In one of the studies, it was found that minimal psychological impact of screening occurred when the system made efforts to reduce risky interpretation of results by the subjects (Kraft et al. 2018). Similarly, while designing a screening program or postscreening counseling, one should always consider different negative scenarios, which can occur due to wrong testing, interpretation, or counseling strategies. Thus, designing screening programs in a way that minimizes pressure, stigma, psychological impact, anxiety, interpretation of results, and provides an individual the choice to refuse, remains a challenge (Ross 2006).

20.4 Genetic Counseling Adoption By Clinics

Counseling is one of the important pillars of screening as it helps people to understand what screening is all about, why it should be taken, and what can be done if tested positive. There is a continuous debate on the type of counseling and how it must be offered to avoid challenges faced by the counselor. Genetics experts in the USA pointed out that some of the limitations associated with counseling included false positives, ambiguous results, and the potential misunderstanding of negative results (Cho et al. 2013). Thus, participants must have their consent along with better result interpretation and appropriate counseling (Cho et al. 2013).

Making educated decisions about the disease to be evaluated was a significant issue during pretest counseling, according to Janssens et al. 2017, who spoke with 16 European geneticists. They asserted that obtaining a subject's consent for a specific ailment that is being tested was highly impracticable and thus demanded a straightforward method of describing diseases based on their characteristics (Janssens et al. 2017). From a survey of North American genetic counselors, it was found that a similar concern of having better approaches during counseling and their tools is required (Lazarin et al. 2016). Rothwell et al. (2017) on interviewing 17 pregnant women who tested positive for expanded carrier screening found that they were confused and could not differentiate between carrier screening and prenatal testing (Rothwell et al. 2017). They also experienced emotional uncertainty as a result of the different ways in which the results were explained (Rothwell et al. 2017). Therefore,

good counseling is very important for the success of carrier screening programs and their adoption.

The practices of clinicians, the availability of numerous testing choices, the complexity of case management, and the ambiguity surrounding the detection of mild mutations, complex alleles, and variants of questionable significance were problems during cystic fibrosis carrier testing. Therefore, emphasis must be placed on training nongenetics experts, standards for increased consistency in test provision, expert resources for continuing genetic counsellor education, and research on the relationship between genotype and phenotype (Wellington 2012). One should be more careful while providing expanded carrier screening test counseling as it includes various diseases, and it is quite difficult for a counselor to explain all of them to the subjects. According to a survey of 272 Australian Jewish students who received expanded carrier screening, they had lower knowledge than those who were screened for a single disease (Ioannou et al. 2010).

Due to societal norms, such as consanguinity or termination of pregnancy, in countries like Saudi Arabia, it is even more difficult for counselors to help people with their reproductive choices. Another challenge faced during genetic counseling is requesting the fiancé of a carrier female to undergo screening which might violate ethical principles; thus, counselors have to be more cautious as they might breach some of the moral principles (Balobaid et al. 2016). Some countries do also face a shortage of genetic counselors, and their access is quite challenging. In the state of Indiana, the National Genetic Counsellor Organization is working on increasing the workforce, mainly in direct patient care (Foil et al. 2019). In a study on Fragile X syndrome (FXS), counselors reported having trouble finding the time to describe the screening procedure and dealing with FXS's heterogeneous phenotype and psychological effects as well as its complex inheritance pattern. Educating obstetricians, midwives, and counselors, along with distributing pamphlets to pregnant women, might help overcome a few of these challenges (Mak and Leung 2017).

20.5 Technical Knowledge About Tests and Genetic Makeup of Populations

There are limitations to the knowledge of sequencing techniques for some conditions and populations that are to be screened. For example, a technical challenge exists in sequencing large structural alterations and pseudogenes (SMN1 in spinal muscular atrophy). Along with this, the knowledge to interpret the sequence of the variants, and the interpretation of new associated variants that might be linked to pathogenicity, is also required (Kraft et al. 2019).

In-depth knowledge of sequencing is required as some variants are pathogenic to some groups while the same variant may not be pathogenic to another group. For example, pathogenic variants of CF in the non-Hispanic Caucasian population are not accountable for the disease in other populations, varying from 48% in Asian Americans to 94% in the Ashkenazi Jewish population (Watson et al. 2004; Palomaki et al. 2004). Therefore, it is very important to have technical knowledge

about DNA sequencing to view different variants that might affect or result in pathogenicity (Watson et al. 2004; Palomaki et al. 2004).

While talking about expanded carrier screening that involves a large window of variants to be screened, having technical knowledge becomes even more important as it will help in identifying novel variants with unknown phenotypes among the populations for which genetic information is scanty. Adoption of new techniques for carrier screening or advancements in screening techniques is a major challenge that varies across populations and countries. The availability of professionals knowing contemporary techniques that can identify the variant and counsel subjects is also a major problem. The cost-effectiveness of most genome sequencing applications is also one of the challenges in the adoption of screening programs (Krier et al. 2016).

20.6 Ethical and Legal Challenges

While performing carrier screening, numerous ethical and legal issues should be addressed. Issues including consent, reproductive autonomy, privacy discrimination, confidentiality, and pregnancy termination pose significant challenges (Potter et al. 2009). In the case of expanded screening panels, there is a regular increase in diseases that are included; therefore, valid informed consent is not taken due to information overload. The lack of patient consent was seen in countries like the UK, USA, and Australia where women were not aware that they are being screened for thalassemia unless they are being tested positive (Cousens et al. 2010). Being informed at a later stage in testing might breach ethical principles. Thus, it is important to inform people about the test they are being offered and why it is being offered, which will help in creating further awareness and gaining individual consent at the same time (Cousens et al. 2010).

Another ethical concern is a negative screening result that might give false assurance of no risk, which might be due to the test's incomplete sensitivity (https://www.ajmc.com/view/clinical-utility-and-practical-implications-of-carrier-screening). Various challenges that reproductive screening includes eventually create ethical pressure on clinicians. Testing positive might result in discrimination against the individual already suffering from a disease, and genetic discrimination is one of the biggest ethical issues (Grady 1999). Genetic makeup is a private matter related to an individual's identity, whose confidentiality is very important as it affects an individual's right to make reproductive choices. The disclosure of testing results can be an issue affecting and hampering the rights of an individual (Grady 1999). Legal issues might arise from ethical issues when the concepts of autonomy and anonymity are breached. Therefore, ethical issues must be taken care of to avoid any legal implications arising from screening programs (Andrews et al. 1994). Ethical issues in reproductive carrier screening are discussed in detail in Chap. 18.

20.7 Conclusions and Future Perspective

Reproductive carrier screening has improved in upgrading from single disease to multiple diseases, from specific ethnic groups to population-based screening, and technical advancements in molecular techniques. Apart from inherent challenges in carrier screening, these advancements may pose further challenges that must be addressed. Challenges that usually surface are the lack of knowledge, understanding the importance of genetic diseases among general public, practical challenges faced by counselors during pre–post tests, obtaining informed consent, the voluntary decision of participation, ensuring disability-based indiscrimination, varied ethical and societal issues across populations, the adoption of evolving technology, and the development of adequate infrastructure. Similarly, numerous other challenges need to be taken care of for better implementation and adoption of carrier screening programs. All the above challenges must be overcome or kept in mind while designing any screening program for better implementation.

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